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THE KENNETH C. SWAN

Proctor Medal Award

PROCEEDINGS

of the

Association for Research in Ophthalmology, Inc.

Twenty-second Meeting

New York, New York

June 1, 2, 3, and 4, 1953

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For a complete table of contents see page one

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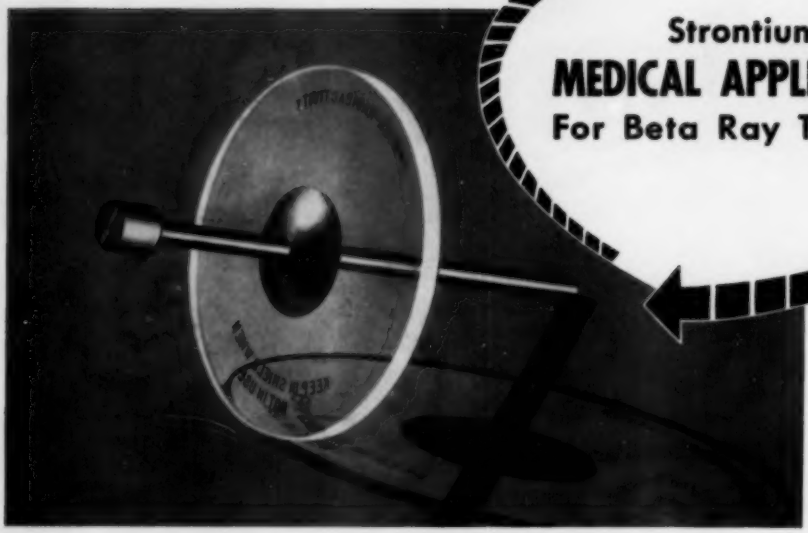

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KENNETH C. SWAN, M.D.

REMARKS MADE ON ACCEPTANCE OF THE PROCTOR MEDAL AWARD

June, 1953

KENNETH C. SWAN, M.D.
Portland, Oregon

When notified that I was to be awarded the Proctor Medal, my thoughts first turned to the contributions of earlier recipients. My work seemed meager by comparison. Then came the realization that in future years it would be relatively unimportant just who received the medal back in 1953. Of far greater significance would be the fact that this unique association met and, to stimulate research in ophthalmology, again honored the contributions of an individual.

Members of this association come from private offices and institutions, medical schools, universities, and colleges. Each in his own way is seeking to advance ophthalmology and my way will continue to be through the medium of a medical school.

What is the place of research in a department of ophthalmology?

At the University of Oregon Medical School the ophthalmologic faculty feels that research should not be simply an isolated activity of a few dedicated men. Instead, we have hoped to make research a part of the every-day learning of ophthalmology.

I have been inspired by my teachers, notably Harold B. Myers and Cecil S. O'Brien, to believe that, by learning, more is meant than self-education of an individual. There are at least three levels:

First, there is the beginning student who strives through classwork and disciplined study to assimilate the knowledge which has been gathered by his predecessors.

If he reaches a faculty status, his learning mechanism advances to a higher level. It has been said that to teach is to learn twice, but in a rapidly advancing field like ophthalmology, it is more than that. A good teacher in ophthalmology constantly must be reviewing, sifting, and reorganizing facts and data in the light of his own experience. As he does this, his knowledge grows and also becomes more applicable to still another means of education.

The highest level seems to be achieved when an adequately prepared individual investigates unexplored regions for new truths. Then he does more than assimilate or disseminate knowledge. He contributes to it.

Research activity seems the most advanced approach to learning, yet it need not be deferred until an individual is advanced in his education. At the University of Oregon Medical School, a unique system of student assistantships in the basic-science departments has been in effect for many years.

Each year a few students with interest and aptitude participate, on a part-time salaried basis, in departmental teaching and research programs. These students have the opportunity to broaden their education beyond the scope of the regular medical school curriculum and, thereby, to become better physicians.

As a sophomore student, I was fortunate

to be appointed a student assistant in the department of pharmacology. My major research project concerned the effects on the pupil of pressor extracts from the pituitary.

The work had little scientific significance but conducting it profoundly altered my approach to the study of medicine. In an educational sense, I began to mature because I was no longer merely assimilating the teachings of others. The process of education by investigation had started. Furthermore, as I worked on this little problem in ocular pharmacology, the first vistas of ophthalmology and ophthalmologic research began to appear before me.

I am convinced that there is a great opportunity to attract outstanding research-minded young people into ophthalmology by the establishment of student assistantships in ophthalmology departments. In 1946, through a donation by Dr. Frank B. Ralston, it was possible to initiate such a program at Oregon.

Each year, our department has given some financial support and an early introduction to ophthalmology to an outstanding medical student. At least three of these students have chosen ophthalmology as a career. Several have come to us with advanced degrees in basic sciences and have been capable of originating, as well as participating in, ophthalmologic research projects.

I am aware that the incorporation of original investigation into the every-day activities of a department has not met with universal approval. Some concern has been expressed that comprehensive programs of research are leading to overspecialization and isolation of ophthalmology. I believe this fear to be unfounded. My experience has been that, as investigations delve deeper into ophthalmology, they also become increasingly fundamental. Then the investigator in ophthalmology finds himself on a common ground for discussions with physiologists, biochemists, and others in the basic-science fields. The membership list of this association establishes this as a fact. Relationships with other clinical departments also are closer because the fundamental problems of cellular physiology and pathology are common to all fields.

Fear also has been expressed that the increasing emphasis on research will make physicians so preoccupied with the scientific aspects of disease that they will forget that the patient is an individual. In reality, this seems to occur least often when physicians have been research minded. This is so because, when a physician is seeking new clues to the nature of disease processes, he does more than is required merely to satisfy the patient's complaints.

As Dr. Friedenwald pointed out in 1948, clinical research in ophthalmology to be fruitful must be diligent for "the crop has largely been harvested." This diligence almost invariably increases the contacts of the research-minded ophthalmologist with the individual and his problems.

It has been my experience, then, that individual investigation can be an important part of learning ophthalmology and that, rather than leading to isolation, research leads to closer contacts with patients and colleagues in all fields of medicine and science.

Investigative work by its nature cannot be conducted on a routine schedule. More time to work seems a need in every ophthalmology department. I believe that time for research is best obtained by utilizing an investigative approach to both teaching and care of patients; however, some think that, if men in academic ophthalmology were relieved of administrative burdens, there would be additional time for research, teaching, and care of patients. I cannot concur with this and am concerned because it seems to be becoming more prevalent. In at least two of our new medical schools, the administration of ophthalmology has been placed under general surgical divisions and students are graduating without have been in an eye clinic.

As the head of a department, even a small one, I have a diversity of daily time-consuming administrative tasks which include staff appointments, budgets, and reports. I have

learned to perform these tasks willingly because with them goes the basic responsibility for the conduct and future of ophthalmologic activities in the school.

Furthermore, if there is to be sound administration of ophthalmologic activities within the framework of institutional policies, the ophthalmology faculty must take an active interest and participate in the affairs of the whole institution. Only then can ophthalmology deserve to retain its proper place in medical education and only by retaining this place can special facilities and trained personnel be obtained for conducting ophthalmologic research.

The special laboratories, clinics, and hospital facilities required for teaching and research in ophthalmology never have seemed possible without the striving of individuals with great interest and insight into the particular problems of our special field. Fortunately, Dr. Francis I. Proctor was an individual of this type. The new laboratory in ophthalmology at the University of California will be dedicated to him. It is a great honor to be the recipient of a medal given in his memory. I thank the Association for Research in Ophthalmology for bestowing it upon me and sincerely hope that the future will justify this action.

BIOGRAPHICAL DATA

Kenneth C. Swan, M.D.

Dr. Kenneth C. Swan, professor of ophthalmology at the University of Oregon Medical School, was born January 1, 1912, in Kansas City, Missouri. His family moved to Oregon when he was a small child. He received his bachelor's degree from the University at Eugene, and his medical degree from the University of Oregon Medical School in Portland. For three years he was a student assistant in pharmacology, working under the late Dr. Harold B. Myers. While a medical student, he completed the first of many studies in ocular pharmacology and was elected to Alpha Omega Alpha and Sigma Xi, honorary medical and scientific societies.

Dr. Swan interned at the University of Wisconsin General Hospital, Madison, and served a residency in ophthalmology under Dr. C. S. O'Brien at the State University of Iowa. While still a resident, he completed, with W. M. Hart, a study of the influence of autonomic drugs on the blood-aqueous barrier. This was the prize-winning paper at the 1940 meeting of the Association for Research in Ophthalmology.

After completing his basic training, Dr. Swan continued under Dr. O'Brien, first as a research associate, and then as assistant professor. He concentrated his pharmacologic investigations on the physical properties of drugs and drug vehicles, and he was the first to add methylcellulose and surface-active agents to drug vehicles to facilitate tissue contact and penetration. With N. G. White he synthesized a new class of choline-like esters which block the stimulatory effects of acetylcholine. The actions of these drugs proved his thesis that physical properties influence the mechanism of action of drugs as well as effect their penetration and distribution into the tissues. At Iowa, Dr. Swan also became interested in the sensory aspects of the physiology and pathologic physiology of binocular vision. He made extensive investigations of retinal correspondence.

In 1945, Dr. Swan was appointed professor at the University of Oregon Medical School. It became his aim to organize an integrated program of research and teaching in ophthalmology. A first step was to establish an ophthalmologic laboratory. This was made possible by the support of Dr. and Mrs. John E. Weeks. Another great need in the Pacific Northwest was a graduate program in ophthalmology which would include teaching in the basic sciences. The ophthalmology

residency at the University of Oregon Medical School was started in 1945. Later it was integrated with the service at the U. S. Veterans Hospital in Portland.

In 1949, with support of the Oregon State Elks Association and in co-operation with the Oregon State School for the Blind, a Children's Eye Clinic was established as a center for teaching and research in pediatric opthalmology.

Dr. Swan has continued to conduct investigative work. In the field of binocular vision he has been particularly interested in the role of the physiologic blindspot in the alleviation of diplopia in patients with esotropia. In connection with this interest he has served as a member of the American Orthoptic Council, as a consultant on ophthalmic devices for the American Medical Association, as a member of the American Committee on Optics and Visual Physiology, and as chairman of the Strabismus Symposium sponsored by the American Academy of Ophthalmology and Otolaryngology.

Dr. Swan also has continued his work in ocular pharmacology and ophthalmic pathology. His Proctor Lecture concerned the pharmacology and toxicology of the cornea. His Charles May Memorial Lecture concerned the classification of scleral disease based on underlying pathology rather than on clinical appearance. In the interest of advancing research he has served as trustee for the Association for Research in Ophthalmology, as a member of the Sensory Diseases Study Section of the U. S. Public Health Service, and on the Editorial Board of the *A.M.A. Archives of Ophthalmology.*

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CLINICAL ASPECTS OF PATHOLOGIC CHANGES IN THE VITREOUS BODY*

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Boston, Massachusetts

INTRODUCTION

For the present, the only methods by which the vitreous body can be examined in vivo are optical methods, with focal or slitlike illumination. An ordinary slitlamp microscope is not a completely satisfactory instrument for this purpose, because it is impossible to focus it on the posterior portion of the vitreous body.

Two groups of attempts have been made to overcome this difficulty. In the first, a telescopic instrument is used, which permits focusing on objects located further away than the distance at which they can be seen with a low-power microscope. This is the principle of ophthalmoscopy with focal or slitlike illumination, and was first described by Wolff[43] in 1901; it was later modified by several authors, particularly Friedenwald[14] (1928), Zamenhof[44] (1930), Evans[12] (1932), and Lopez Enriquez[27] (1935).

The advantage of this method is that it is optically simple. The disadvantages are that the instruments lack versatility, the beam of light often has insufficient sharpness of contour and intensity, and it is difficult to vary the angle formed by incident and emergent rays.

In a second type of attempt, a low-power microscope has been used, such as the one available with any slitlamp. The devices employed so far made it necessary to neutralize the refractive power of the patient's eye. One way to accomplish this is to apply the principle of the hydrophthalmoscope, which was first described by Mery,[28] in 1704 and explained by De la Hire,[8] in 1709. This idea was developed into a more practical instrument by Czermak[7] (1854); Coccius[5] described the first flat contact lens; and Koeppe,[22] in 1918, designed the contact lens still in use nowadays for examining the vitreous body and retina with a slitlamp. Another procedure for neutralizing the refractive power of the patient's eye consists of placing a highly concave lens close to the cornea. This method was first described by Stilling[35] (1879) and was later developed by Lemoine and Valois[25] (1923) and by Hruby[19] (1950).

The attractiveness of the flat contact lens and of the concave precorneal lens results from the fact that these methods permit using the slitlamp microscope, which is a readily available instrument with good focal and slitlike illumination, and with an adequate low-power microscope. One difficulty, however, is that in the ordinary slitlamp the angle formed by the axis of illumination and the axis of observation is too large.

Many devices have been proposed to reduce this angle: Koeppe[22] used a front silvered mirror (1918); this was modified by Gallemaerts and Kleefield[15] (1922), by Lacarrere[24] (1925), by Evans[13] (1932), and by Lopez Enriquez[27] (1935). Lindner[26] recommended using an angulated monocular microscope (1936); but Goldmann[16] (1937) designed a "reduction prism" by means of which a binocular view can be obtained. In the latest (1950) slitlamp model of Zeiss-Oberkochen (Germany), and of Aimark (England), the angle may be varied from 0 to 90 degrees without using additional optical

* From the Retina Foundation and Retina Service, Massachusetts Eye and Ear Infirmary, and from the Department of Ophthalmology, Harvard Medical School. This work was supported by the Institute of Neurological Diseases and Blindness, of the National Institute of Health, Public Health Service (Grant #B-56R and B-56C), by the Vivian B. Allen Foundation, and by the American Optical Company.

parts; this is a definite advantage over the reduction prisms, because it avoids certain disturbing reflections.

I have made a careful comparison between the results obtained with the precorneal lens, supplied by Haag-Streit, and with a flat contact lens of the Koeppe type, made especially for this study;* each method has advantages and disadvantages. The precorneal lens is easier to handle and gives a somewhat better view of that portion of the vitreous body which is located close to the equator of the globe. The extreme periphery of the vitreous body cannot, however, be seen by this procedure. With the flat contact lens, there are fewer reflections, and this makes observation of the posterior pole easier: when examining this area, the image is sharper than with the precorneal lens. Finally, the magnification and the field of view are larger than with the precorneal lens.

All methods of clinical investigation of the vitreous body have so far been hampered by several limitations which should be recognized. Firstly, one should remember that vitreous structure is best visible when examined on a dark background. In order to obtain a dark background, it is necessary that the axis, along which the observer examines the vitreous body, should make a relatively large angle with the axis of the illuminating beam of the slitlamp. In other words, incident and emergent rays should form a relatively large angle. This condition can only be fulfilled when the pupil is dilated and when the portion of the vitreous body under examination is anteriorly located. The part of the vitreous body which is close to the retina can only be examined against the reddish background of the choroid. As a consequence, in this region, it is often extremely difficult to make out details of structure.

Another important limitation is the impossibility of examining the extreme periphery of the vitreous body; this is not inconsistent with the fact that the edge of a retinal dialysis may be observed by this method, whenever it has been pushed considerably toward the center of the globe by subretinal fluid.

In spite of these two important limitations, the procedure is at present the most valuable clinical method for study of the vitreum in vivo.

Anatomic Attachments of the Vitreous Body

Before discussing pathologic changes observed in the vitreous body, its normal attachments should be described because the location and morphology of many fundus changes, caused by the vitreum, are directly related to these attachments. The anatomic details which follow have been checked by careful dissection of 12 normal human eyes, recently enucleated, and examined before fixation.

Anteriorly, the vitreous body is attached to the posterior lens capsule, in a circle some nine mm. in diameter (Wieger[41]). Adhesions to the zonule and ciliary body are weak and easily ruptured by blunt dissection. The vitreo-retinal symphysis, or base of the vitreous body (Salzmann[37]), occupies an area about two mm. broad, extending over the posterior half of the pars plana ciliaris and over the ora serrata. This is the main attachment of the vitreous body to the retina and it is well known that attempts to separate it from the rest of the globe, at the symphysis, always result in tearing off shreds of epithelium from the pars plana ciliaris. The anterior limit of the symphysis is in the middle of the pars plana ciliaris, and is sometimes marked either by a pale gray line or by an irregularly pigmented line. The strongest of the zonular fibers are also attached along this line (Wolff[42]). The posterior limit of the symphysis coincides with the posterior border of the ora serrata.

It is important not to confuse the symphysis with the anatomic attachment of the

* This flat contact lens was made by the American Optical Company, Southbridge, Massachusetts.

retina to the pigment epithelium, at the ora serrata (fig. 1); the latter attachment will henceforth be called the retinochoroidal attachment. Its posterior limit coincides with the posterior border of the ora serrata; it occupies a band-shaped area which shows fairly sharply limited and dense choroidal pigmentation.

On the temporal side, this pigment band and the corresponding retinochoroidal attachment are 1.5 to 2.0 mm. broad; there are reinforcements of the retinochoroidal attachment along the temporal striae ciliares, which are pigmented lines extending from the ora serrata to the valleys between the ciliary processes.

On the nasal side, the retinochoroidal attachment and its corresponding pigment band are very narrow and follow exactly the scallops of the ora serrata. As a result of this anatomic arrangement, the retinochoroidal attachment is strong on the temporal side and much weaker nasally; in the latter region, therefore, it is easy to detach the retina from the pigment epithelium.

The posterior border of the retinochoroidal attachment coincides everywhere with the posterior limit of the vitreo-retinal symphysis. The anterior border approximately coincides with that of the symphysis on the temporal side; nasally it reaches the anterior limit of the symphysis only at the tip of the teeth of the ora serrata.

In many cases, abnormal vitreo-retinal adhesions are observed, posterior to and continuous with the symphysis; they are generally much weaker than the symphysis itself, partly because they are made of separate small areas of adhesion. The posterior limit of these abnormal adhesions varies, of

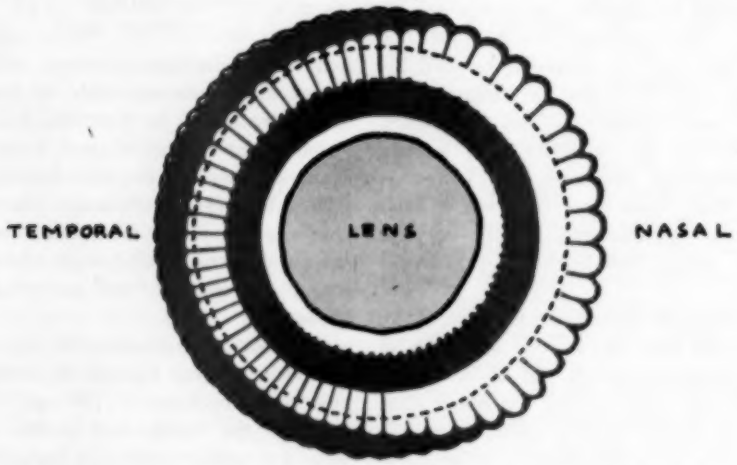

Fig. 1 (Schepens). Schematic drawing of the anterior segment of a human globe, seen from the back. The lens is surrounded by the corona ciliaris (ciliary processes). The vitreo-retinal symphysis, or vitreous base, occupies a band about two-mm. broad: its anterior border is indicated by a dotted black line and its posterior border coincides with the scalloped line of the ora serrata (continuous black line). The area of attachment of the retina to the pigment epithelium is represented in red. In the region of the pars plana ciliaris, this attachment is narrow and follows the scallops of the ora serrata on the nasal side, whereas temporally it is wide and coincides approximately with the vitreo-retinal symphysis. On the temporal side, there are reinforcements of the attachment along the striae ciliares which are pigmented lines running from the ora serrata to the valleys between the ciliary processes. Nasally, it is uncertain whether or not such reinforcements exist in the anterior portion of the pars plana ciliaris. The area of the pars plana ciliaris which corresponds to the retinochoroidal adhesion (in red) is markedly pigmented. Over the ciliary processes, the ciliary epithelium is strongly adherent to the pigment epithelium.

course, with the case, and in the same eye it may vary according to the quadrant considered. In several eyes dissected, it reached a line 10 to 12 mm. posterior to the ora serrata; the presence of abnormal vitreoretinal adhesions could not be detected except by actual dissection of the area involved.

It seems that, normally, weak adhesions between vitreous body and retina are present in the equatorial region of the globe; this confirms an observation made earlier by Grignolo,[17] who has also described a firm anatomic adhesion between vitreous body and retina in the macular area; a similar but somewhat stronger adhesion to the disc has been described by many authors. In the region of the disc and of the macula, it is difficult or impossible to separate the vitreous from the retina without breaking the surface layer or hyaloid membrane.

Pathologic changes in the vitreous body

Various pathologic changes observed clinically in the vitreous body have been described by a number of authors such as Hruby,[19] Koby,[21] Vogt,[39] and others. Many fruitless attempts have been made to detect changes in the vitreous body which were pathognomonic for eye conditions such as myopia, senility, retinal detachment, inflammatory condition, trauma, old hemorrhage, and so forth.

Clinical experience and a survey of the literature[2, 11, 19, 21, 25, 39] have convinced me that degenerative changes in the vitreous body may vary greatly in appearance, but few or none have been shown to be characteristic of any one of the above conditions.

It is probable, therefore, that the vitreous body reacts in a similar fashion under the influence of a number of different injurious agents, and consequently, it appears futile to elaborate on an etiologic classification of its disturbances. In the present state of our knowledge, the pathologic changes observed in the vitreous body may be divided into three main groups (table 1).

The first group contains cases showing embryonic remnants. In the second group, the pathologic process results from an invasion of the vitreous cavity by elements which are foreign to the vitreous body itself; such elements may either be exogenous, such as parasites or foreign bodies, or endogenous. The endogenous elements are either essentially cellular, such as blood, connective and glial tissue, or acellular, such as pigment, exudates, crystals of cholesterol (synchisis scintillans), or calcium soaps (asteroid bodies).

In the first and second groups, the pathologic changes are mostly histologic in nature and lend themselves well to study by standard histologic methods.

In the third group, which contains altera-

TABLE 1

Pathologic changes in the vitreous body

1. Embryonic remnants
2. Invasion by foreign elements (causing histologic changes)
   - A. Exogenous: parasites, foreign bodies
   - B. Endogenous:
     - a. Cellular: blood; connective tissue; glial tissue
     - b. Acellular: pigment; exudates; cholesterol; calcium soaps
3. Alterations of the vitreous gel (causing physicochemical changes)
   - A. Syneresis
   - B. Membranes
   - C. Coarse fibers
   - D. Shrinkage

tions of the vitreous gel, the changes observed may be divided as follows: syneresis, appearance of membranes, appearance of coarse fibers, and shrinkage of either part or the whole vitreous body. These changes are not essentially different from those observed in a great number of other gels; they do not lend themselves to histologic study, because the lesions are essentially physicochemical in nature.

There is no condition which is known to produce specifically any one of these changes in the vitreous gel; apparently they may all result from a number of different causes. In the following pages, I will discuss clinical aspects of the physicochemical changes observed in the vitreous gel.

There is not always a clear-cut difference in the optical aspect of normal and pathologic vitreous gel in vivo; for instance, normally, it often contains frontally disposed loose membranes, visible with the slitlamp; it also frequently contains loose fibers. Senescence tends to make membranes and fibers more conspicuous. At the same time, partial syneresis is frequent in the old, even in the absence of any visible ocular pathology. It seems, therefore, that pathologic syneresis, membrane formation, and formation of coarse fibers are only exaggerations of normal phenomena. It is not known whether or not a moderate amount of shrinkage of the vitreous body may occur as a physiologic phenomenon.

Syneresis

Syneresis is a phenomenon by which the vitreous gel liquefies. Optically, liquefied vitreous is like aqueous, in that it appears empty when traversed by the light beam of the slitlamp. There are cases, however, in which the liquefied portion still contains either visible loose fibers, or floating particles similar to those seen in the anterior chamber, in case of uveitis (fig. 2). Some light is reflected at the area of transition between the gel and the liquid phase, which gives the impression that a fine membrane with little mobility separates the two phases.

Fig. 2 (Schepens). Slitlamp view of an eye with total retinal detachment; the retina is represented by a thick and wavy white line. The central portion of the vitreous gel has undergone syneresis; the optically empty pocket contains glittering corpuscles analogous to those found in the anterior chambers in cases of uveitis. A fine but easily visible membrane lines the optically empty pocket; it may conceivably be formed by the lining of Cloquet's canal but it corresponds neither to the shape nor to the exact location of this canal. The pocket is attached to the retina above the disc where it creates a marked fixed retinal fold. It is likely that this fold is, in part at least, the result of gravity; vitreous gel in the upper half of the globe probably causes traction on the attachment, to the retina, of the lining of the optically empty pocket.

No vitreous gel is represented in contact with the retina because it was impossible to make sure of either its presence or its absence in this area.

This picture was observed in a man, aged 25 years, who, 13 years previously, sustained a contusion of the eye which reduced his vision to 20/200 and caused an iridodialysis. The patient noticed a sudden and almost total loss of vision two weeks before examination. No remarkable abnormalities were detected in the other eye (T. B., Retina Service No. 1487).

The tendency to syneresis may be somewhat related to the fact that the composition of the vitreous gel is not uniform. The protein and mucopolysaccharide molecules, which form the gel, are most concentrated in the cortical zone of the vitreous body (Balazs[2]); it is therefore reasonable to assume that the gel will resist syneresis better at the periphery than in the center.

Consequently, when the tendency to syneresis is approximately uniform over the whole vitreous body, it is probable that the central portion liquefies first, and is surrounded by a more resistant peripheral crust of vitreous gel.

I have observed this in two freshly enucleated human eyes, opened before fixation. The globes showed no visible pathologic changes; the vitreous body, however, was completely fluid, except for a layer about one-mm. thick overlying the retina, ciliary body, and lens. Irvine[20] describes a similar observation in one of 12 fresh adult human eyes.

In many cases, the tendency to syneresis is not uniform through the whole vitreous body, but it is more or less elective over areas where the choroid, retina, or ciliary body show more marked pathologic processes. As vitreous gel has a slightly higher specific gravity than liquefied vitreous, partial syneresis may cause a pull on the upper insertions of the vitreous body, by simple gravity (fig. 2). Consequently, it may cause a type of pathology which is similar to that resulting from shrinkage of the vitreous body, and which will be discussed at the end of this paper.

Membrane formation

A second important phenomenon is the appearance of membranes in the vitreous cavity. It has already been mentioned that syneresis of a portion of the vitreous gel gives the impression that a delicate membrane separates the liquid from the gel phase. In many pathologic conditions, one or several condensation membranes appear in the vitreous gel; this phenomenon is nothing more than the exaggeration of a normal tendency which becomes more marked with advancing years.

Pathologic condensation membranes are often observed after contusion of the globe, after vitreous hemorrhage, after inflammation of the posterior segment, and in degenerative diseases (fig. 3). Sometimes, vitreous

Fig. 3 (Schepens). Slitlamp view of an eye showing four wavy membranes and one taut membrane in the vitreous cavity. On the most posterior of the wavy membranes there were numerous small clots of blood, which disappeared a few months later. The posterior limit of the visible vitreous gel was formed by a taut membrane, of the same optical density as the posterior lens capsule. It could not be decided whether or not this membrane was the hyaloid, because it was impossible to determine whether or not vitreous gel was present in contact with the retina. The latter, represented by a thick white and wavy line, showed a flat retinal detachment with a sharp fold in the vicinity of the equator. This fold resulted from traction exerted by the taut membrane; traction was probably also the cause of the vitreous hemorrhage observed in this case. Eight months later, the three most anterior wavy membranes became invisible and the retinal detachment increased.

This observation was made in the left eye of a man, aged 62 years, who had five diopters of myopia in both eyes. Two years previously, a spontaneous retinal detachment with three horseshoe-shaped breaks had been diagnosed in this eye, which was operated on successfully. The above picture was seen shortly after a spontaneous vitreous hemorrhage of moderate amount; no new retinal break was found after the hemorrhage cleared. No remarkable changes were observed in the right eye (R. P., Retina Service No. 919).

gel of approximately equal optical density is present on both sides of a membrane (fig. 4); in other cases, however, the gel looks less dense on one side.

There are also eyes in which an optically empty space is present on one side of a membrane, but not on the other (figs. 2, 3, and 5). In the latter case, one often speaks of "vitre-

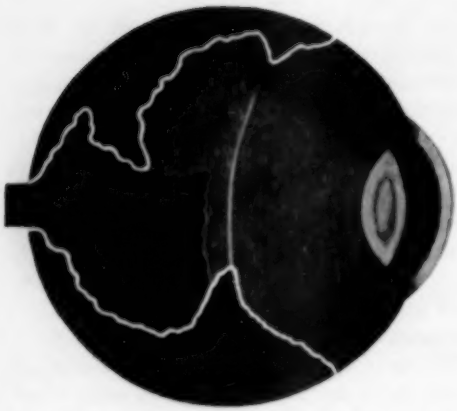

Fig. 4 (Schepens). Slitlamp view of an eye with total retinal detachment of long standing. The retina, represented by a thick and wavy white line, forms multiple fixed folds. A smooth, taut membrane is visible in the vitreous cavity; it has the optical density of the posterior lens capsule. The taut membrane causes a fixed retinal fold in the equatorial region, and this fold is much larger in the inferior half of the fundus. Behind the smooth membrane, vitreous gel is still visible. The gel is limited by a thinner membrane, which is probably the hyaloid, because vitreous gel is seen in contact with the retina above the disc, but not below. In this case, therefore, one may assume the presence of a true inferior vitreous detachment. Brown pigment deposits are visible on the horizontal portion of the thin membrane.

This observation was made in the left eye of a man, aged 57 years, who had six diopters of myopia. A retinal detachment had been present for four years. The right eye showed a subclinical retinal detachment with the appearance of a vitreous detachment (E. C., Retina Service No. 2074).

ous detachment," an expression which implies that the membrane separating the optically empty space from the vitreous gel is the hyaloid membrane.

In a number of cases, there are signs indicating that the membrane observed is really the hyaloid. When, for instance, it shows an annular opacity in front of the optic disc, this is generally interpreted as being formed by peripapillary glial tissue which has been torn off when the attachment of the vitreous body to the optic disc was torn. Inside the annular opacity there is a hole in the limiting membrane and, sometimes, solid vitreous is seen to herniate through this hole. Quite frequently the annular opacity is continuous with a denser area extending temporally, in the hyaloid membrane; in this area, it is not rare to see a second hole in the hyaloid, round in shape, smaller than the one in front of the disc, and representing the torn attachment of the hyaloid membrane to the macula.

Similar observations were made by a number of authors; Rieger,[34] for instance, mentions that holes may be observed not only in front of the disc and macula but also in

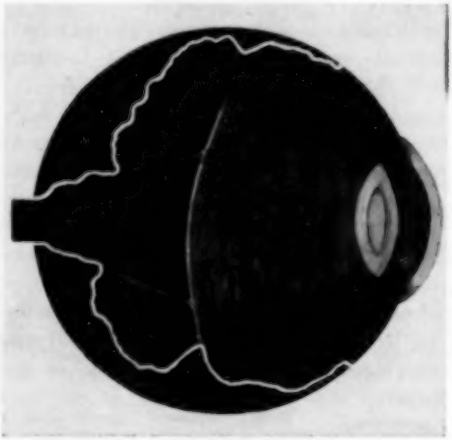

Fig. 5 (Schepens). Slitlamp view of an eye with total retinal detachment and multiple fixed folds (the retina is represented by a thick and wavy white line). The vitreous gel seems limited posteriorly by a smooth and taut membrane, which has an optical density equal to that of the posterior lens capsule, and causes an equatorial fixed retinal fold; the latter is more marked below. Posterior to the taut membrane is an optically empty space, except for two coarse strands which connect the retina with the smooth membrane, causing two additional fixed retinal folds above and below the disc. As it is impossible, in this case, to decide whether or not vitreous gel is in contact with the retina, it is uncertain whether the taut membrane is really the hyaloid or not; therefore, it is not safe to call this a vitreous detachment.

This observation was made in a man, aged 68 years, whose vision became blurred in the left eye two weeks previously. The right eye showed a thin and wavy membrane in the superoposterior portion of the vitreous cavity (C. C., Retina Service No. 1394).

the periphery. Rare cases have been observed where the retina was torn at the macula, with an operculum attached to the hyaloid membrane (fig. 6). In one case, neither hyaloid membrane nor retina became torn, but both were elongated in a bizarre fashion, a long strand pulling on a tubular-shaped macular area.

It is my opinion that, unfortunately, in most cases of so-called vitreous detachment, it is impossible to tell with certainty whether or not one deals with the hyaloid membrane (figs. 2, 3, and 5). In fact, there are good reasons, which will not be developed here, for thinking that in many instances the hyaloid membrane is still in place and that the appearance is one of pseudodetachment of the vitreous body; the later could result either from the formation of a membrane in the vitreous cavity or from partial synaeresis without formation of a real membrane.

Membranes found in the vitreous cavity vary considerably in optical density, and also in mobility. Extremely mobile membranes appear to be of sinuous contour and of low optical density; immobile membranes often seem to be under stretch and are generally of high optical density.

An interesting type of dense and immobile membrane is that which sometimes stretches across the vitreous cavity, dividing it in two along the equatorial plane of the eye (figs. 3, 4, and 5). It is generally taut and may show traction folds; in some cases, holes are seen in it, which seem to correspond to the former area of attachment to the disc and macula, but in many cases such holes are not found. Vogt[39] has suggested that a single hole in this location may be caused by the passage of the hyaloid canal through the membrane.

In a few cases, vitreous gel was seen posteriorly to the membrane. In one case (fig. 4), a dense, taut, equatorial membrane was seen, posterior to which was vitreous gel, itself limited, farther posteriorly, by what seemed to be the hyaloid membrane. The latter appeared less dense, optically, than the equatorial membrane. It seems certain, therefore, that in some cases, at least, this taut, equatorial membrane is not the hyaloid. In many of the cases observed, the optical density of an equatorial membrane appeared equal to, or higher than, the posterior lens capsule.

The attachment of a dense and taut equatorial membrane to the retina is nearly always visible, as it creates a sharp equatorial ridge; the latter, as a rule, is more pronounced below and it may not be visible all around the equator. Subretinal hemorrhages and exudates are sometimes found in the vicinity of the ridge in the retina; at a later stage, chorioretinal degeneration occurs and the lesions observed then simulate an ordinary quiescent chorioretinitis. Retinal tears, often of large size, may be produced, and they are frequently accompanied by bleeding into the vitreous cavity.

Coarse fibers

Fibers in the vitreous body, which are visible by slitlamp examination, have a very polymorphous appearance; for instance, they may be arranged in irregular bundles like tangled skeins of wool, or like threads of gauze floating in the liquefied vitreous gel. Sometimes, fibers are seen to course in the portion of the vitreous cavity where no gel is visible (fig. 5). When they appear to be under tension, they may cause important pathologic changes.

Fibers under stretch are often attached to the retina and, in case of retinal detachment, they are a cause of fixed retinal folds, which are similar to those resulting from traction by a membrane. When the retina is detached and shows multiple fixed folds, the latter may be produced by the combination of a taut membrane and tense coarse fibers in the vitreous cavity (fig. 5).

It has occasionally been observed that fixed folds in the retina resulted from traction by many faintly visible taut fibers. This type of fiber can be produced experimentally in freshly enucleated, normal human eyes by

traction on the vitreous, close to a vitreo-retinal adhesion. It seems, therefore, that the appearance of faintly visible fibers in the vitreous gel is a reversible optical phenomenon resulting from localized traction.

In a number of cases, many faintly visible taut fibers, or a fairly dense fibrous strand, are attached to the tongue-shaped flap of a retinal tear or to an operculum. Another type of pathologic change is due to traction by fibers.

It is unknown whether or not there is any relationship between clinically visible fibers in the vitreous cavity and the fibers which Grignolo[17, 15] described in fresh, normal human vitreous, with the phase-contrast microscope.

Shrinkage

Shrinkage of either part or the whole vitreous body is one of the most destructive phenomena occurring in the vitreous gel. In fact, one might say that most of the changes described so far: syneresis, membrane formation, and appearance of fibers, are relatively innocuous as long as they are not associated with shrinkage. For instance, the severity of the complications attending the formation of a taut equatorial membrane appears to depend entirely upon the amount of shrinkage of this membrane.

The action of gravity may be associated with shrinkage and increases the pathologic changes observed. It is probable that gravity is responsible for the relative frequency with which certain lesions resulting from traction, for instance retinal breaks, occur in the upper part of the fundus. In cases where partial syneresis is present, gravity alone may be responsible for changes ascribed to shrinkage (fig. 2).

It is proposed to discuss briefly how shrinkage and gravity may affect the normal and abnormal attachments of the vitreous body to the retina; traction on the normal attachments may cause signs and symptoms which will be described first, and traction on abnormal vitreo-retinal adhesions will be considered next.

Traction on the attachment of the vitreous body to the optic disc is sometimes a cause of elevation of the disc margins, which may be called pseudopapilledema. The disc margins are not particularly hazy, but may be elevated as much as three or four diopters; small hemorrhages may be present on the disc, but there are generally no exudates and no hemorrhages around it. This phenomenon often precedes a separation of the hyaloid from the region of the optic disc; it may also conceivably be the cause of edema of the optic disc, which occasionally follows intraocular surgery.

If shrinkage of the vitreous gel causes traction on the macular area, metamorphopsia is caused, with centrally located flashes of light; macular edema, a macular cyst, and a retinal break in the macula may also result. The importance of this factor in the production of macular cysts in general and of other types of hemorrhagic and degenerative macular conditions cannot be determined at present.

Traction on the vitreo-retinal symphysis, in the region of the ora serrata, does not cause photopsiae because this portion of the retina is not sensitive to light. The resulting changes depend upon anatomic relationships between the retinochoroidal attachment and the vitreo-retinal symphysis, which have been described earlier in this paper. When no retinal detachment is present, chronic irritation of the retinochoroidal attachment, by constant traction, may be the cause of chorioretinal degeneration; the degenerated zone is always broader on the temporal side because the retinochoroidal attachment is broader temporally (fig. 1).

When retinal detachment is present, traction on the vitreo-retinal symphysis may produce a retinal fold which is slightly posterior and runs parallel to the ora serrata. In some cases, a second fold is present along the anterior border of the vitreo-retinal symphysis in the middle of the pars plana ciliaris.

Traction on the nasal portion of the vitreo-retinal symphysis causes meridional folds to form in the retina because the retinochoroidal attachment is scalloped nasally.

At a more advanced stage, the attachment of the retina to the choroid, along the ora serrata, may be torn off. Ophthalmoscopic examination of this region then gives the impression that there is a double outline to the ora serrata: the most posterior line corresponds to the ora serrata and the anterior line is a fold in the middle of the pars plana ciliaris. As the retinochoroidal attachment is weaker nasally, detachment of the ora serrata from the choroid is more frequent on the nasal side.

Traction by the vitreous body in the region of the symphysis also plays an important role in the production of retinal dialyses and of other retinal breaks in the region of the ora serrata, but this subject will not be discussed here.

Traction on abnormal vitreo-retinal adhesions will now be considered. When adhesions have extended posteriorly to the symphysis, they may become an important cause of pathology in the region located between the ora serrata and the equator. Experimentally, traction on such adhesions causes tears in the retina at or near the posterior limit of the adhesion.

Clinically, the frequent occurrence of retinal breaks near the equator may be partly explained on the basis that the posterior limit of abnormal vitreo-retinal adhesions is often located near the equator. Traction in this region nearly always causes photopsiae, which generally affect an extensive area of the patient's visual field,[4] and are much more frequently observed in the temporal field, because the nasal periphery of the retina is more sensitive to light. When extensive light flashes are observed in the temporal field, a so-called vitreous detachment is frequently visible and small retinal hemorrhages may be found in the periphery of the fundus, close to the posterior limit of the vitreo-retinal adhesion.

It results from what was said previously, that the syndrome of light flashes associated with a vitreous detachment may be the result of three different processes. Firstly, the whole vitreous body may shrink, causing the hyaloid membrane to detach from the retina and to pull upon vitreo-retinal adhesions; secondly, partial syneresis of the vitreous gel may occur and the action of gravity upon the remaining portion of the gel may cause photopsiae; finally, a membrane or coarse fibers may form in the vitreous body and light flashes may be produced by their traction upon the retina.

If there is a strong and isolated abnormal vitreo-retinal adhesion in one point, traction may cause localized photopsiae, which often precede the formation of a retinal tear. As the pathogenesis of retinal tears is complicated by other factors, it will not be discussed further here. Transient flashes of light may be caused by weak, abnormal vitreo-retinal adhesions which break off without tearing the retina.

Vitreous hemorrhage is known to be a frequent and early sign of retinal detachment; it nearly always results from the rupture of a retinal vessel at the point where a retinal tear forms. The hemorrhage and retinal tear result from traction on a localized area of the retina. When the vitreous hemorrhage is profuse, and retinal tears are present, a taut equatorial membrane is frequently found to be the underlying cause.

Comments

A rapid survey of the literature shows that, among the changes observed in the vitreous gel, the appearance of membranes has been investigated in more detail than any other alteration. As early as 1858, Mueller[39] described a case of detachment of the vitreous body from the retina, in a pathologic specimen. The first clear description of a clinical case is probably that of Dimmer[10] (1887). In the last 50 years there have been many clinical observations published, some of the most recent articles on the subject

being those of Adamantiadis,[1] Costi,[6] De Vries,[9] Pischel,[22] and Posner.[23]

Many authors have assumed that any membrane observed in the vitreous cavity was nearly always the hyaloid membrane; they have noted only very briefly some of the diagnostic difficulties which result from either partial syneresis or formation of condensation membranes in the vitreous gel (Sallmann and Rieger,[25] Hruby,[19] and others).

The concepts outlined in this article, relative to the origin of membranes in the vitreous cavity, are not in absolute agreement with some of the views held by authors of previous publications. For instance, Hruby[19] feels that the hyaloid membrane may be so thin that it is almost invisible; in his opinion, a diagnosis of vitreous detachment may be made, almost with certainty, simply by detecting the presence of well-formed vitreous opacities in front of the posterior pole, with a concave mirror. I feel that the diagnosis of vitreous detachment should be made somewhat more cautiously.

Vogt[29] has indicated that an annular opacity may form in the vitreous cavity without real detachment of the hyaloid membrane; Sallmann and Rieger[25] have observed annular opacities which were not connected with any membrane. This appearance is most frequently observed in myopic eyes. Moeschlin Sandoz[29] has described the annular prepapillary opacity as being formed of glial tissue, which has been torn away from the peripapillary region by the shrinking vitreous gel.

This process does not necessarily cause a separation between the hyaloid membrane and the retina. It must be admitted, on the other hand, that the hyaloid membrane does not always have a marked optical density.

In a few cases in which this membrane was most probably detached from the retina, because shreds of retinal tissue were still attached to it, it was only visible as a very slight film. One could argue, therefore, that an annular opacity which seems to float freely may, in fact, be attached to an invisible hyaloid membrane; in cases of this kind, the diagnosis of vitreous detachment should perhaps not be made unless there is some other clear indication in its favor.

Several authors state that the space between the retina and the detached hyaloid membrane is not always optically empty (Hruby,[19] and others). According to them, it may contain material of an optical density slightly lower or almost equal to that of vitreous gel. It is my opinion that it is not really justified to make a diagnosis of detachment of the hyaloid membrane in such cases.

Even if the membrane seen in the vitreous cavity shows one or two holes, located in the region of the posterior pole, the diagnosis of vitreous detachment cannot be made indiscriminately.

There are several types of membranes with holes which may be seen in the vitreous cavity, and which are certainly not the hyaloid. For instance, perforated membranes are fairly frequently observed in the vitreous cavity in cases of chorioretinitis. Another type of membrane was described by Wagner[40] and by Vogt[29]: it consists of a thick hyaline sheet, coated with glial cells and perforated by several round or oval holes.

Finally, a word of caution should be expressed with regard to conclusions drawn from examination of the vitreous gel in fixed material. The vitreous gel contains 98.7-percent water and it is evident that the dehydration caused by fixation alters considerably the physicochemical state of the gel. A vitreous detachment found in a fixed specimen is often an artefact.

It is commonly said that the presence of a coagulum, between a membrane in the vitreous cavity and the retina, indicates that the membrane was not in contact with the retina before fixation. This is not certain, as shrinkage of the hyaloid membrane could well cause an artificial vitreous detachment with escape of vitreous gel through holes in the membrane. In all cases, an existing

vitreous detachment becomes markedly exaggerated by the fixation process (Pillat,[31] Sallmann[36]).

On the strength of examination of fixed material only, Rieger[34] describes a type of detachment of the vitreous where the hyaloid membrane is smooth and separated from the entire retina, only being attached at the ora serrata. This observation requires further confirmation in clinical cases and in fresh human specimens.

Examination of the vitreous body before fixation, in freshly enucleated eyes, was probably performed first by Sallmann;[36] it is to be hoped that this valuable procedure will be used on a large scale in order to study more thoroughly pathologic changes in the vitreous gel.

Summary

The most practical method devised so far for examination of the vitreous body in vivo is the use of the slitlamp with either a flat contact lens of the Koeppe type or a highly concave precorneal lens of the Stilling type. No procedure permits satisfactory examination of that portion of the vitreous body which is located in the vicinity of the choroid.

The main attachment of the vitreous body to the retina is the vitreous base, which is a band two-mm. broad, going from the ora serrata to the middle of the pars plana ciliaris. The relationships between the vitreous base and the attachment of the retina to the pigment epithelium, in the region of the ora serrata, are described. Secondary attachments of the vitreous to the retina are found in the region of the disc, of the macula, and perhaps also at the equator.

Pathologic changes observed in the vitreous body may be divided into embryonic remnants, invasion by foreign elements, and alterations of the vitreous gel. The latter are subdivided into syneresis, membrane formation, coarse fiber formation, and shrinkage; they form the subject of this paper. There is not always a clear-cut difference between physiologic and pathologic changes in the vitreous gel.

Syneresis is a phenomenon by which either part or the whole vitreous gel liquefies. There are indications that the tendency to liquefaction is less marked in the peripheral portion of the gel than in the central part. Syneresis may be accompanied by pathologic changes induced by gravity.

Of all the changes observed in the vitreous gel, membrane formation has so far attracted the greatest amount of attention. This change is often called vitreous detachment, which implies that the membrane seen in the vitreous cavity is the hyaloid.

Signs which help to identify the hyaloid membrane are: the presence of a hole surrounded by an annular opacity in front of the disc; a similar, but smaller hole, in the membrane in front of the macula; the presence of an operculum of retinal tissue attached to the membrane.

In many cases, however, it is impossible to say whether or not the membrane seen is really the hyaloid. Of interest is the occasionally observed dense, taut membrane which divides the vitreous cavity in two portions and is attached to the retina near the equator; in many cases its origin is uncertain.

Fibers in the vitreous cavity are very polymorphous. When under stretch, they often create isolated fixed retinal folds. Not uncommonly, they are attached to a membrane in the vitreous cavity. The appearance of faintly visible, taut fibers is a reversible optical phenomenon resulting from localized traction on the vitreous gel.

Shrinkage is the most destructive phenomenon occurring in the vitreous gel. At the posterior pole it may cause pseudopapilledema, macular edema, a macular cyst, or a macular break. Traction on the vitreo-retinal symphysis causes chronic irritation which results in chorioretinal degeneration confined to the region of the symphysis. When retinal detachment is present, traction on the vitreo-retinal symphysis causes a ret-

inal fold on either side of the ora serrata and parallel to it; nasally, it causes the formation of meridional folds and detachment of the retina from its choroidal attachment. Traction on abnormal vitreo-retinal adhesions may cause photopsiae, retinal breaks, and bleeding into the vitreous cavity.

30 Chambers Street (14).

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STUDIES ON THE STRUCTURE OF THE VITREOUS BODY*

I. The absorption of ultraviolet light

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The ultraviolet absorption of the pig vitreous was studied by Graham[1] in 1923. His experiments indicated that there was a strong absorption at the shorter wavelengths because no transmission occurred below the 2,265 Å Cd line. In the same year, Shoji[2] observed that below 2,978 Å there was a definite absorption maximum in the vitreous body and that this maximum varied with the hydrogen ion concentration. In 1927, Abe[3] made a detailed study of the absorption of cattle vitreous. He found that the vitreous has an absorption band at 265 mμ which could not be identified with the absorption band of proteins. The ultraviolet absorption of different eye tissues was studied by Kinsey[4] in 1946. He concluded that the vitreous absorbed most of the radiation of wavelengths shorter than 300 mμ.

Experimental

Preparation of vitreous body

Cattle eyes collected within one hour after the slaughter of the animal were stored for two or three hours at +4° C. They were then carefully separated from muscle and connective tissues, washed in physiologic saline, and dried with a cloth. The eyeball, resting on the posterior part of the sclera, was opened by an equatorial cut with a Stadie-Riggs tissue slicer.[5]

The anterior part of the vitreous attached to the ora serrata was discarded. The posterior part of the vitreous was divided into two parts: a central and a cortical portion.

The division was accomplished by inserting a stainless steel cylinder with a sharp cutting edge into the vitreous to a point three to five mm. from the retina, and lifting out the central part of the vitreous with a stainless steel baller before removing the cylinder. This portion will be referred to as the "central sample."

Two sizes of cylinders were used (19 mm. or 25 mm. in diameter), depending on the size of the eyeball. When the central sample was being taken out, care was taken to leave a three- to five-mm. thick cortical layer of vitreous at the bottom of the cylinder. The cylinder was removed and the rest of the vitreous gel was lifted out with curved forceps. This part of the vitreous will be referred to as the "cortical sample." Both samples were removed in the gel state from the posterior part of the eye; liquid vitreous was never included in the samples.

* From the Retina Foundation, Massachusetts Eye and Ear Infirmary and the Department of Ophthalmology, Harvard Medical School. This investigation was supported by the National Council to Combat Blindness, Inc., New York, Grant No. 107, and by a research grant, Grant No. B56R, from the National Institutes of Neurological Diseases and Blindness, Department of Health, Education, and Welfare.

The samples were checked for possible "contamination" by membranes, fibers, and foreign tissues in the following way: The cortical sample and the central sample were placed in separate Petri dishes and observed under a strong narrow beam of light directed to the bottom of the dish at an angle of 50 to 70 degrees. Occasionally, a five-power magnifying glass was used to enable closer scrutiny of the sample. When viewed under this condition, the cortical sample showed membranous structures attached to the surface of the gel (hyaloid membrane), while the central sample was free from any visible structures.

If any tissue elements such as blood, pigment, or retinal tissue were observed in either sample, it was discarded. These two samples could be easily obtained from steer eyes, but it was difficult to prepare a central sample free from fibrous and membranous structures from cow eyes.

Following the separation procedure, the gels were liquefied in one of two ways. One method consisted in placing the gel in a syringe and forcing it through a series of hypodermic needles of decreasing size from gauge 16 to gauge 25. In the other method the gel was centrifuged in a Spinco preparative ultracentrifuge (Model L) for 30 minutes at 17,000-20,000 × G.

Preparation of aqueous humor

The aqueous humor was collected in a syringe by inserting a hypodermic needle through the cornea into the anterior chamber. The collected aqueous was centrifuged in a Spinco Model L preparative ultracentrifuge at approximately 13,000 × G.

Dialysis of samples

Both samples were dialyzed against buffer solutions in an Aminco mechanical dialyzer* to remove the low molecular weight components.†

* American Instrument Company, Incorporated, Silver Spring, Maryland.

† Nojax Cellulose Casings, The Visking Corporation, Chicago, Illinois.

Absorption measurements

Absorption of the ultraviolet light was measured with a Beckman Model DU photoelectric spectrophotometer. The transmittance values of the instrument were checked with a potassium chromate standard according to a method described by G. W. Haupt.[6] The wavelength scale of the spectrophotometer was calibrated according to Gibson and Balcom.[7] Quartz cells with a one-cm. light path were used. For measuring solutions with higher optical density without dilution, precision quartz inserts‡ were placed in the quartz cell reducing the light path to 0.1 or 0.05 cm.

Measurement of hydrogen-ion concentration

A Beckman Model G pH meter with a glass electrode, Model 290, was used to measure pH between 1 and 11.

Nitrogen and hexosamine determinations

Nitrogen was determined according to the micro-Kjeldahl method with selenium, potassium sulfate, and copper sulfate catalysts.[8] Hexosamine was determined after the Elson-Morgan method modified by Blix.[9] One ml. of dialyzed vitreous sample was

‡ Pyrocell Manufacturing Company, New York, New York.

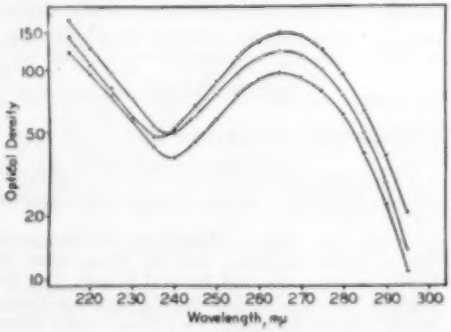

Fig. 1 (Balazs). The absorption spectra of cattle vitreous and aqueous. ●–●: Central sample of vitreous. ○–○: Cortical sample of vitreous. ◑–◑: Aqueous humor.

hydrolyzed with four ml. of 2N HCl at 96°C. for 16 hours.

Results

Undialyzed vitreous

Both the central sample and the cortical sample measured in the gel state immediately after removal from the eye showed an absorption spectrum with two bands between 200 and 300 mμ (fig. 1). One absorption band had a maximum below 200 mμ. The other band had a maximum at 265 mμ with the minimum point between the two bands at 240 mμ. At 265 mμ the optical density, varying with individual eyes, was between 10 and 15. The optical density of the cortical sample was in all cases somewhat higher than that of the central sample. Liquefaction did not change the absorption spectrum of the vitreous gel. The absorption spectrum of the aqueous humor in the ultraviolet was similar to that of the vitreous samples (fig. 1).

Dialyzed vitreous

Dialysis of both samples of the vitreous against distilled water or against a pH 7.6 phosphate buffer, ionic strength 0.12, led to

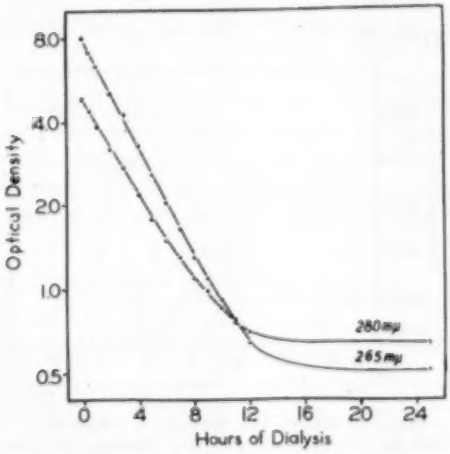

Fig. 2 (Balazs). The decrease of density at 280 and 265 mμ during dialysis of vitreous body (central sample).

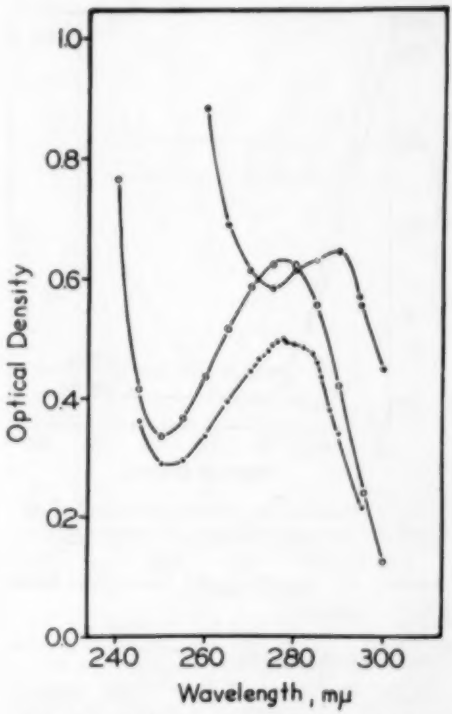

Fig. 3 (Balazs). The absorption spectra of dialyzed vitreous and aqueous at different pH values:
●–●: Central sample of vitreous, pH 6.0 (maximum at 277 mμ).
◑–◑: Central sample of vitreous, pH 10.0 (maximum at 290 mμ).
○–○: Aqueous humor, pH 6.0 (maximum at 277 mμ).

a profound change in the absorption spectrum. During dialysis the optical density at 265 mμ dropped rapidly, and after 12 hours a new band developed with a maximum at 277 mμ (fig. 2). The absorption spectrum showed a second band under 220 mμ with a maximum below 200 mμ. The minimum point between these two bands occurred at 250 mμ (fig. 3). Both absorption bands remained constant during storage at +4° C. for several weeks.

The same rapid decrease in optical density was observed with the dialysis of aqueous humor (fig. 4). The absorption spectrum of the dialyzed aqueous was similar to that of the dialyzed vitreous samples (fig. 3). Acidi-

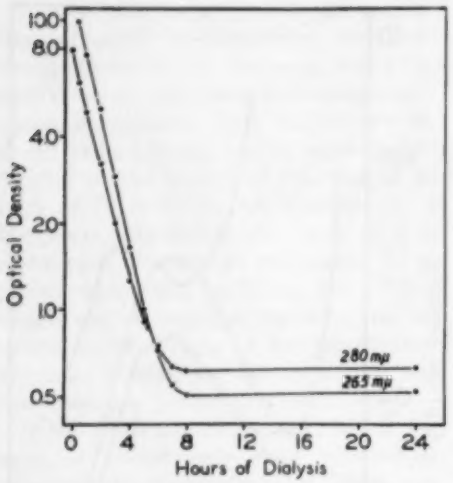

Fig. 4 (Balazs). The decrease of density at 280 and 265 mμ during dialysis of aqueous humor.

fication did not affect the absorption bands of these samples.

VITREOUS DIALYSATE

The vitreous samples were dialyzed against 0.1 normal HCl for 24 hours. After dialysis, the pH was adjusted to 6.0 using 0.1 N sodium hydroxide. The absorption bands between 200 and 300 mμ showed the same characteristics as the undialyzed vitreous, namely maximum at 265 mμ, minimum at 240 mμ and end absorption under 220 mμ. Acidification of the vitreous dialysate with hydrochloric acid below pH 5.0 caused a gradual shift of the absorption band to the shorter wavelengths. At pH 3.0, the maximum moved to 245 mμ. No further shift could be observed below this pH (fig. 5). The optical density of the vitreous samples below pH 3.0 was very stable, but above pH 5.0 it gradually decreased during storage.

The samples of vitreous dialysate were adjusted at room temperature to pH 1.8 and pH 6.3. The optical density at 245 mμ in the pH 1.8 sample did not change significantly during a period of 120 minutes. The optical density at 265 mμ of the pH 6.3 sample dropped, during the same period of time, to the level of the dialyzed vitreous (fig. 6), and the absorption maximum at 265 mμ disappeared.

With 10 μg/ml. KCN the stability was considerably increased between pH 6.0 and 7.0. If hydrogen peroxide or copper sulfate was added to it, the density decreased rapidly, and the maximum at 265 mμ disappeared, but the end absorption remained unchanged. With acidification the aqueous humor dialysate showed the same kind of gradual shift of the absorption maximum as the vitreous dialysate.

ULTRAVIOLET ABSORPTION AT HIGH PH VALUES

If sodium hydroxide was added to the vitreous dialysate to raise the pH above 8.0, the density values decreased for the whole range of the absorption spectrum between 200 and 300 mμ and the maximum shifted to 290 mμ. This new absorption band, formed under pH 10, was not very stable, and in 20 minutes the peak flattened out to a plateau.

If sodium hydroxide was added to the dialyzed vitreous so that the pH value was increased above pH 7.0, no change could be observed until pH 10.0 was reached. At pH values higher than 10.0 the absorption maximum shifted to 290 mμ and the optical

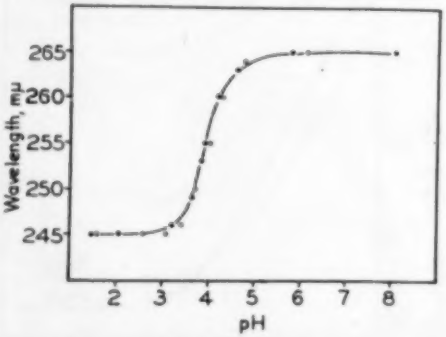

Fig. 5 (Balazs). The position of the absorption maxima of ascorbic acid and vitreous dialysate plotted against different pH values adjusted with HCl and NaOH: ○–○: Ascorbic acid (10 mg. percent). ●–●: Vitreous dialysate (central sample) 1:1 dilution.

density was increased (fig. 3). This newly formed band in contrast to that of the vitreous dialysate was very stable and the shift was reversible with re-acidification.

Spectrophotometric determination of tyrosine and tryptophane

Holiday[10] has shown that it is possible to analyze mixtures of tryptophane and tyrosine quantitatively by means of their ultraviolet absorption. Goodwin and Morton[11] recommended a formula, based on this principle, for the determination of the tyrosine and tryptophane content of protein solutions. The optical density of a protein solution in N/10 alkali at wavelengths 2,800 and 2,944 Å together with the nitrogen content of the solution are used for calculating the grams of tyrosine and tryptophane in 100 grams of protein.

The central sample of the vitreous was dialyzed against pH 7.0 phosphate buffer (0.06 Mol.) with 0.1 Mol. NaCl. To 2.0 ml. of vitreous, 1.0 ml. 0.3 N NaOH was added and the optical density measured at the desired wavelengths. The protein content was calculated from the total nitrogen content of the dialyzed samples (6.25 × protein nitrogen) after subtraction of hexosamine nitrogen.

The tyrosine and tryptophane values are shown in Table 1. Sample 1 is the average calculated from seven individually prepared and determined central samples of cattle eyes. Samples 2 and 3 are two different preparations of pooled central sample of cattle eyes. The tyrosine and tryptophane values

TABLE 1

Tyrosine and tryptophane content of proteins in cattle vitreous

	Sample Number	Tyrosine	Tryptophane
mg. per 100 ml. Vitreous	1	3.2	1.4
	2	3.3	1.7
	3	3.2	1.3
gm. per 100 gm. Protein	1	7.0	3.0
	2	7.9	4.2
	3	7.5	3.1

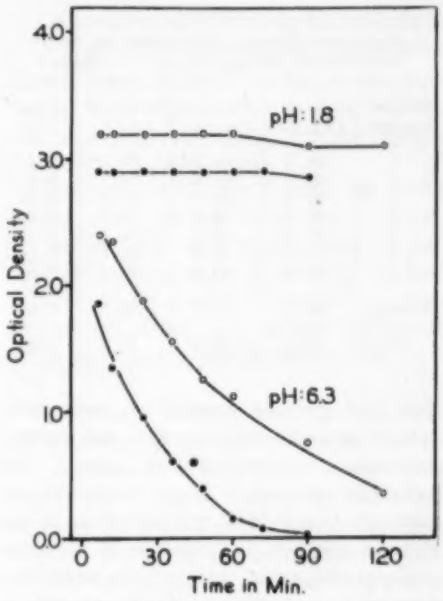

Fig. 6 (Balazs). The effect of storage on the stability of vitreous dialysate and ascorbic acid at pH 1.8 and 6.3: ○–○: Vitreous dialysate (central sample) 1:3 dilution. ●–●: Ascorbic acid, 2.5 mg. percent; pH 1.8—in HCl acid; pH 6.3—in 0.06 M PO_4 buffer.

obtained from the three samples are generally in good agreement except the tryptophane value of Sample 2.

Spectrophotometric estimation of albumin and globulin

F. C. Smith[12] suggested a method of estimating albumin-globulin ratios in serum by measuring the optical density against total protein content, calculated by nitrogen determination. The Lerner and Barnum modification[13] of this method was used, measuring the optical density of the dialyzed vitreous at 2,775 Å in 1.0-cm. layer. The albumin was calculated according to the following equation:

Albumin = 1.5 × total protein (gm. per 100 ml.) − 0.1 × optical density of dialyzed vitreous (see details in Lerner and Barnum). No allowance was made for non-protein absorption. The total protein, albu-

TABLE 2

ALBUMIN AND GLOBULIN ESTIMATION BY SPECTROPHOTOMETRIC METHOD IN CATTLE VITREOUS

Sample Number	Protein (mg.%)	Albumin (mg.%)	Globulin (mg.%)	A/G
1	48.8	14.7	34.1	0.43
2	47.5	13.8	33.7	0.41
3	46.3	18.0	28.3	0.64
4	37.5	10.8	26.7	0.40
5	45.6	14.9	30.7	0.49
6	52.5	18.8	33.7	0.56
7	45.6	10.6	35.0	0.30
Average	46.2	14.5	31.7	0.46
11	42.1	13.1	29.0	0.45

min, and globulin content of seven individual central samples and one pooled preparation are presented in Table 2. The individual variation is larger in the albumin content of individual samples than in the globulin content. The average of the albumin-globulin ratio, 0.46, of the individual samples and of the pooled vitreous sample, 0.45, are in good agreement.

DISCUSSION

The ultraviolet absorption of the undialyzed vitreous is predominantly due to its ascorbic acid content. Baird and others[14] studied the ultraviolet absorption of ascorbic acid and found that above pH 5.0 it has a band with a maximum at 265 mμ. With acidification the maximum shifts to the shorter wavelengths attaining a maximum at 245 mμ at pH 3.0. The shift of the absorption maximum with pH is due to the ionization of the dienol group.

The pK value of ascorbic acid calculated from titration in water is 4.12 according to Kumler and Daniels[15] and 4.17 according to Rosenburg.[16] Daglish found that the pK of ascorbic acid calculated from the shift of the absorption maximum was 4.16.[17] In our experiments the pK value of the vitreous dialysate calculated from the shift of the absorption maximum was 4.16.*

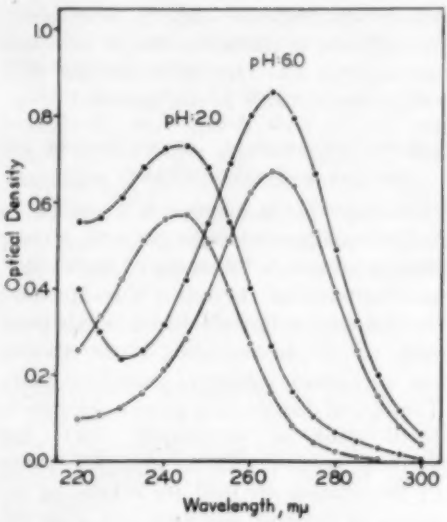

Fig. 7 (Balazs). The absorption spectra of ascorbic acid and vitreous dialysate at different pH values. O—O: 1 mg. percent ascorbic acid. ●—●: Vitreous dialysate. Solvents: pH 2—HCl; pH 6—0.1 M NaCl and 0.01 × 10^{-3} M KCN.

The close agreement of the pK values and the identical character of the absorption spectra in both acid and alkali support the view that the ultraviolet absorption of the undialyzed vitreous is due to its ascorbic acid content (fig. 7). The dialyzability of the absorbing material, its lability in alkali, and in the presence of copper sulfate or of hydrogen peroxide, and its stability in potassium cyanide and in acid solutions, also lend further support to this conclusion.

The ascorbic acid content of the vitreous has been reported on by Johnson.[18] Using

* The dissociation constant in the vitreous dialysate was calculated according to the following formula:

$$K = \frac{(H^+)\ (D^{acid} - D^m)}{D^m - D^{salt}}$$

The density measurements were made in McIlvaine's citric acid-sodium phosphate buffer at three different pH values, namely 1.3, 4.2, and 6.2; the optical density being D^{acid}, D^m, and D^{salt}, respectively.

The calculated pK values were:

4.19 at 255 mμ
4.17 at 260 mμ
4.15 at 265 mμ
4.15 at 270 mμ

Average: = 4.16

the value of the extinction of ascorbic acid given by Baird,[14] we found that the optical density of the vitreous dialysate in the reported experiments would correspond to an ascorbic acid concentration of 12-14 mg. percent. This value is of the same order of magnitude as that given by Johnson.[18]

The absorption band of the dialyzed vitreous at neutral pH is at 2,775 Å and the minimum absorption is at 2,500 Å, indicating that the absorption is due to the protein content. At neutral pH both the bovine serum albumin and serum γ-globulin have an absorption maximum and minimum at those wavelengths. Above pH 10.0 the maximum and minimum of the absorption band shift to the longer wavelengths, and the density values increase. The maximum is at 2,900 Å and the minimum at 2,750 Å at pH 10.0. A similar shift in serum albumin and globulin has been described by several authors who showed that the change was due primarily to the ionization of the phenolic hydroxyl group of the tyrosine residue.[19]

In addition to ascorbic acid and proteins, others substances in the vitreous contribute to its absorption between 200 and 300 mμ. Hyaluronic acid is the most important of these substances. It shows a strong end absorption but no absorption band between 230 and 300 mμ.[20] The possibility of the presence, in very small concentrations, of other absorbing substances, such as uric acid, which occurs in some animal species, could not be completely excluded, but did not show up in our samples.

The absorption of proteins that contain tyrosine change with pH in the 250 to 300 mμ region. This change reflects the spectral shift that accompanies the ionization of the phenolic hydroxyl group. This observation is the basis of the spectrophotometric determination of tyrosine and tryptophane at pH 13.0 in proteins. The determination of these two amino acids by this method is in satisfactory agreement with other analytical data.[19]

The molar ratio of tyrosine to tryptophane is 27.0 in serum albumin and 2.8 in globulins. This ratio is 2.6 in the protein mixture of the dialyzed vitreous. This indicates that some proteins in the vitreous have a high tryptophane content.

Schaeffer and Murray,[23] using the microbiologic assay method for amino acid analysis, found 2.4 percent tyrosine and 3.4 percent tryptophane in the proteins of the total vitreous. In the central sample of the vitreous, we found the same amount for tryptophane and three times the amount for tyrosine.

The value obtained from the albumin-globulin ratio in the serum by spectroscopy was considered to be a truer estimate than that obtained by the salting-out methods.[19] However, in the dialyzed vitreous the reliability of the spectrophotometric determination was limited by the fact that the proteins were not separated and their extinction ($E_1^{1\%}{}_{cm.}$)* was not separately determined. If it is assumed that the $E_1^{1\%}{}_{cm.}$ of the soluble proteins of the dialyzed vitreous is the same as that of the blood proteins, namely, $E_1^{1\%}{}_{cm.} = 5.0$ for albumin and 15.0 for globulins, the albumin-globulin ratio in the vitreous is 0.46. This is a lower value than that found by Cahn[21] in bovine vitreous, 0.75 or by Duke-Elder in horse vitreous, 0.65,[22] both authors having used salting-out methods.

Summary

Cattle vitreous has an absorption band with a maximum at 265 mμ between pH 5.0 to 10.0 which can be attributed to ascorbic acid. The absorption maximum at 277 mμ in dialyzed vitreous is caused by the proteins.

The tyrosine and tryptophane content of

* $E^{1\%}_{1cm.} = \frac{\log I_o/I}{c \times l}$, where c is in g/100 ml and l is in cm.

I am indebted to Dr. J. Gergely for his helpful comments and criticism throughout the course of this work and the preparation of the manuscript. The valuable assistance of Miss Elizabeth Anne Eckl in these experiments is gratefully acknowledged.

vitreous proteins was estimated, and it was found that proteins of the globulin type with relatively high tryptophane content, were present in the greatest quantity.

The albumin-globulin ratio estimated by spectrophotometric methods was 0.46.

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STUDIES ON THE STRUCTURE OF THE VITREOUS BODY*

II. Electrophoretic and sedimentation properties

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Boston, Massachusetts

The first electrophoretic studies on the vitreous were made by Hesselvik.[1] He observed three boundaries in cattle vitreous filtrate. By calculation of mobility values in the pH range 5.5 to 8.0, he concluded that the fastest moving boundary was the "hyalomucoid" fraction which could be precipitated with acetic acid. The slowest component showed the same mobility as the γ-globulin of horse blood serum. The mobility of the middle component differed slightly from that of horse serum albumin.

Blix has isolated the leading component of the cattle vitreous filtrate.[2] He concluded, on the basis of hexosamine and nitrogen determinations, that 90 percent of this component was hyaluronic acid. The redissolved acid precipitate of the vitreous filtrate gave two boundaries. The faster boundary showed the same migration rate as the fastest boundary of the original vitreous filtrate, while the slower boundary had about the same migration rate as the serum albumin.

Blix and Snellman[3] studied the stream double refraction and viscosity of protein-free sodium-hyaluronate prepared from cattle vitreous. They found that, depending on the preparation method, the hyaluronic acid was more or less polydisperse. The length of the particle varied between 2,600 and 4,800 Å in a 1/15 M phosphate buffer of pH 7.0.

In order to understand the gel structure of the vitreous body, it seemed necessary to characterize its components in more detail by physicochemical investigations. This paper presents results of ultracentrifugal and electrophoretic studies of cattle vitreous.

Experimental

Apparatus

Sedimentation velocity determinations were performed with a Spinco analytical ultracentrifuge (Model E) equipped with the Philpot-Svensson cylindrical lens optical system.

Mobility measurements were made with an Aminco portable electrophoresis apparatus also equipped with the Philpot-Svensson cylindrical lens optical system. In all cases the standard analytical cell with 6.0 ml. "protein capacity" was used.

Conductivity measurements were made with an Industrial Instruments, Inc., conductivity bridge, Model RC 16. Mobility and conductivity measurements were made at +0.75° C.

Methods

The ultracentrifugal experiments were run at 52,000 r.p.m. with the sample in buffer medium at +20° C. The sedimentation data given in this paper were not corrected for density and viscosity of the medium, but sedimentation constants used for comparison were obtained from runs made in the same solvents with identical ionic strength and pH. Sedimentation constants were calculated according to the method of Svedberg and Pedersen.[4] Values are given in Svedberg units ($S = 1 \times 10^{-13}$ sec.). Mobility was calculated according to Abramson et al.[5]

A 0.006 M phosphate buffer, adjusted with NaCl to ionic strength 0.12, was used in the pH range between 5.0 and 7.8, and veronal buffer, adjusted with NaCl to ionic strength

* From the Retina Foundation, Massachusetts Eye and Ear Infirmary; the Department of Ophthalmology, Harvard Medical School; and the Department of Biology, Massachusetts Institute of Technology. This investigation was supported by a research grant, Grant No. B56R, from the National Institutes of Neurological Diseases and Blindness, Department of Health, Education and Welfare, and by the Kresge Foundation.

0.12, was used for pH 7.8 to 10.0.

Nitrogen and hexosamine were determined according to methods described in a previous paper.[6]

The dialysis of samples was carried out in cellophane bags* either by agitation of the bag or by stirring its contents.

Proteolysis with crystallized-lyophilized trypsin,† dissolved in phosphate buffer at pH 7.4 and ionic strength 0.12, was carried out at +37° C. with thymol as a bacteriostatic agent. The conditions for the digestion with papain were the same. Hyaluronic acid was digested with bovine testicular hyaluronidase† dissolved in phosphate buffer at pH 7.4 and ionic strength 0.12 and incubated at +37° C. with thymol.

Crystallized bovine serum albumin‡ and bovine serum γ-globulin‡ were used. Hyaluronic acid was prepared according to Blix and Snellman[8] from acetone-dried human umbilical cord. The protein impurity of the preparation was found to be six percent of the dry powder (dried in vacuum at +100° C.) calculated on the basis of hexasamine, hexuronic acid, and nitrogen determinations. The sulfur content was less than 0.1 percent.

Material

Cattle eyes, collected within a few hours after the animals were killed, were stored at +5° C. until used. The posterior central part of the vitreous was prepared as previously described.[6] The gel was liquefied by centrifugation in a Spinco Model L preparative ultracentrifuge for one hour with a relative centrifugal force of over 20,000 × G. The slight sediment was discarded, and the supernatant used for the studies. The material was concentrated by blowing air over filled cellophane dialysis tubing at +5° C. The concentration procedure was interrupted several times with dialysis in order to keep the salt concentration low. The sample was concentrated to one eighth of its original volume without any precipitation. Before analysis the concentrated sample was dialyzed against the appropriate buffer.

In a few cases the vitreous was liquefied in a Waring blendor. The container was surrounded by dry ice so that the temperature of the sample did not rise above +5° C. Foam formation was completely prevented by excluding air during homogenization.

Results

Electrophoresis experiments

Without concentration the liquefied vitreous was not suitable for electrophoretic analysis with the available apparatus since the total protein and mucopolysaccharide content does not exceed 0.1 to 0.15 percent. Consequently, the vitreous used for these experiments was concentrated four to six times after liquefaction.

In a pH 5.0 phosphate buffer only two boundaries were observed even after three hours of analysis. In a pH 8.0 phosphate buffer a third boundary separated distinctly during a three-hour run. At pH 9.8, in veronal buffer, four boundaries became apparent within an hour and these separated completely in three hours (fig. 1). Upon plotting the mobility data of the first three boundaries against pH, the curves shown in Figure 2 were obtained. The very slow moving fourth boundary, appearing only at higher pH values, is not included.

Ultracentrifugal analysis

The gel structure of the vitreous body can be destroyed by centrifugation.[7] If a centrifugal force of 13,000 to 17,000 × G is applied to the vitreous, a gel and a liquid phase separate. Nitrogen and hexosamine determinations of the dialyzed gel and liquid phase showed that the protein and hyaluronic acid content of the liquid phase decreased only by 10 to 15 percent. This

* Nojax cellulose casings, The Visking Corporation, Chicago, Illinois.

† Worthington Biochemical Corporation, Freehold, New Jersey.

‡ Armour Laboratories (Bovine γ-globulin, fraction II). Chicago, Illinois.

Fig. 1 (Varga and Balazs). Electrophoretic pattern of cattle vitreous. Ascending limb. Migration from left to right. (a) Phosphate buffer, pH 5.0, μ 0.12; after 103 minutes at 12 ma. (b) Phosphate buffer, pH 8.0, μ 0.12; after 180 minutes at 12 ma. (c) Veronal buffer, pH 9.8, μ 0.12; after 100 minutes at 10 ma.

indicates that under the influence of centrifugal force a liquefaction, not a shrinkage of gel, occurs. With increased centrifugal force the gel liquefies completely, and the breakdown is irreversible; no regelation or thixotropy can be observed.

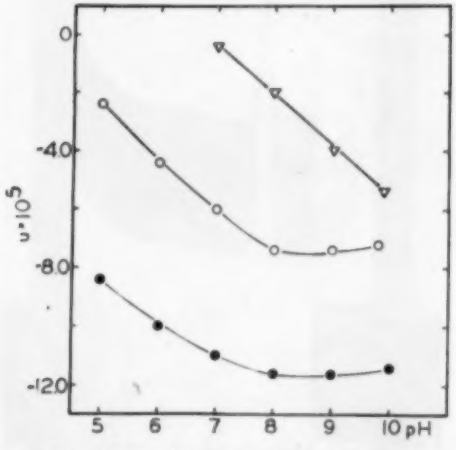

Fig. 2 (Varga and Balazs). Electrophoretic mobilities of the first three boundaries of cattle vitreous at different pH values. Solid circles: first boundary. Open circles: second boundary. Triangles: third boundary.

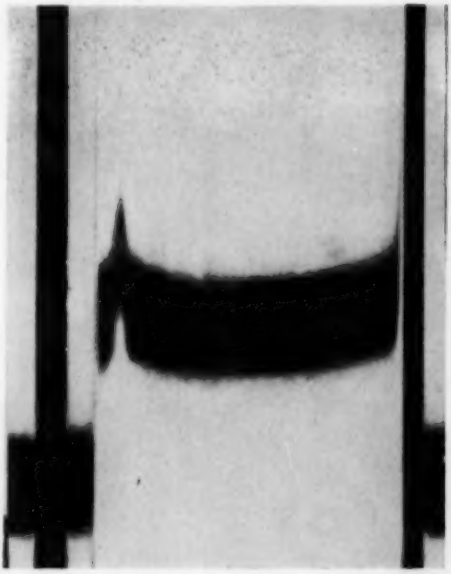

Fig. 3 (Varga and Balazs). Ultracentrifugal pattern of unconcentrated vitreous. Exposure made after 27 minutes. Bar angle: 45 degrees, sedimentation from left to right.

The liquefied vitreous, dialyzed against pH 7.5 phosphate buffer, showed a single boundary which sedimented very slowly (fig. 3). This component gave a value of $s = 2.45$ S. The five-times concentrated vitreous, after dialysis against pH 7.5 phosphate buffer, gave two boundaries. The slower moving one, represented by a sharp, slowly diffusing peak, yielded a value of $s = 0.95$ S. The faster moving component gave a flat, rapidly diffusing peak with a value of $s = 4.10$ S (fig. 4). In the 4.5-times concentrated vitreous in pH 9.12 veronal buffer, ionic strength of 0.12, the same sedimentation pattern could be observed. The slow boundary showed a value of $s = 1.00$ S and the fast moving boundary one of $s = 4.35$ S (fig. 5).

Lyophilized vitreous gel was dissolved in one-fifth volume of distilled water* and dialyzed against a pH 7.5 phosphate buffer.

* There was a small insoluble residue.

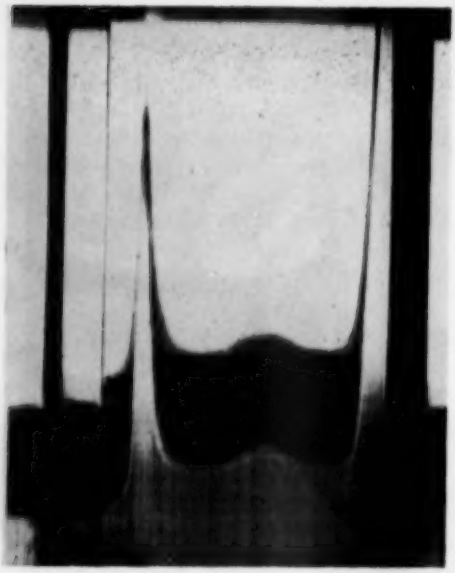

Fig. 4 (Varga and Balazs). Ultracentrifugal pattern of vitreous in pH 7.5, phosphate buffer. Bar angle: 55 degrees, sedimentation from left to right.

The ultracentrifugal pattern was similar to that of the concentrated, unlyophilized vitreous (fig. 6).

The vitreous gel was homogenized with Waring Blender, five-times concentrated and dialyzed against pH 7.5 phosphate buffer. The ultracentrifugal pattern of this preparation was the same as that of the concentrated non-Waring blended vitreous.

Identification of components

In an attempt to identify the components of the vitreous, as revealed in ultracentrifugal analysis, two series of experiments were conducted. In the first, enzymatic treatment was used (trypsin, papain, and hyaluronidase); in the second, known substances were added to the vitreous (hyaluronic acid, albumin, and γ-globulin).

In pH 7.5 phosphate buffer, five-times concentrated vitreous, digested with trypsin or papain, shows only one component, and this is the slow sedimenting, slowly diffusing sharp peak with a value of $s = 0.90$ S (fig. 7). Thus, the fast sedimenting peak which disappeared must have represented the protein component. In the pattern of four-times concentrated vitreous, treated with hyaluronidase, the appearance of the slow moving boundary is altered. Instead of the very characteristic sharp peak a low and broad peak could be observed with a slower sedimentation value of $s = 0.47$ S. No change was observed in the fast moving boundary (fig. 8).

With the addition of 0.15 percent hyaluronic acid to the vitreous only the slow moving boundary was increased. No new boundary appeared (fig. 9-d). A similar type of boundary could be observed when the same hyaluronic acid preparation alone was centrifuged (fig. 9-a). There was no significant difference between the sedimentation constant of the pure hyaluronic acid and the slow moving boundary of the vitreous with added hyaluronic acid. In Figure 9-d the slow boundary was extremely small

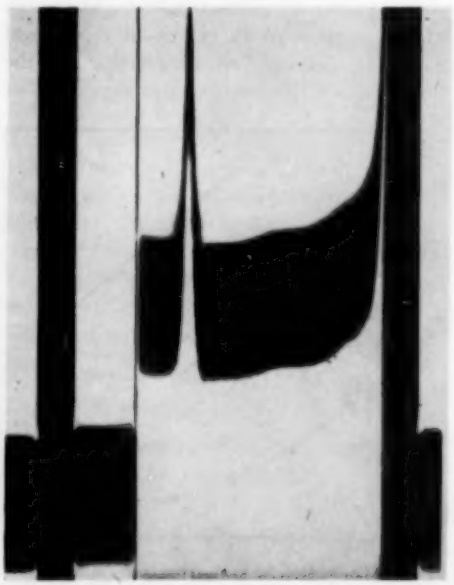

Fig. 5 (Varga and Balazs). Ultracentrifugal pattern of vitreous, pH 9.12, veronal buffer. Exposure made after 120 minutes. Bar angle: 45 degrees, sedimentation from left to right.

because the bar angle was kept high in order to keep the whole hyaluronic acid boundary on the photographic plate.

When serum albumin and hyaluronic acid were mixed in concentrations of 0.2 and 0.15 percent, respectively, two distinct boundaries could be observed (fig. 9-b). On addition of albumin to four-times concentrated vitreous, the slow moving boundary did not change, while the fast-moving boundary increased (fig. 9-e). The sedimentation constants did not change significantly.

When hyaluronic acid, albumin, and globulin were mixed in concentrations of 0.15, 0.2, and 0.2 percent, respectively, these three components showed three distinct boundaries with $s = 1.35$ S, 4.55 S, and 6.75 S values, respectively (fig. 9-c). In our experiments the pure albumin and pure γ-globulin, under the same conditions and concentrations, showed $s = 4.60$ S and 6.50 S, respectively. With the addition of γ-globulin to the concentrated vitreous, only two distinct boundaries could be observed (fig. 9-f). The sedimentation constant of the slow moving boundary was slightly increased to 2.01 S and the fast moving boundary had a typical globulin value of 6.50 S. Even after a run of 83 minutes at 52,000 r.p.m. no third boundary could be clearly observed. There was an indication of a third boundary between the hyaluronic acid and globulin peaks which may represent the albumin component of the vitreous (fig. 9-f).

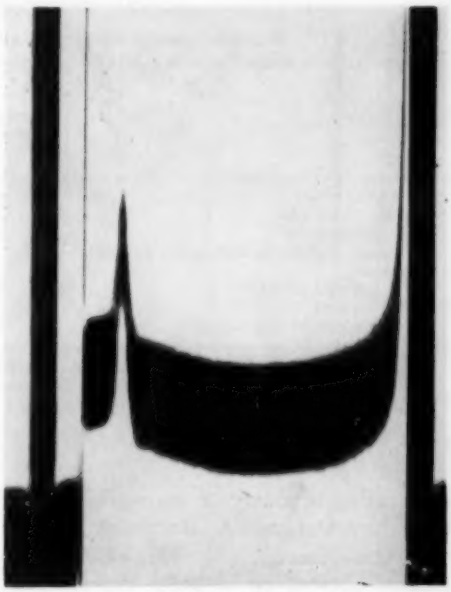

Fig. 7 (Varga and Balazs). Ultracentrifugal pattern of vitreous treated with trypsine. Exposure made after 88 minutes. Bar angle: 50 degrees, sedimentation from left to right.

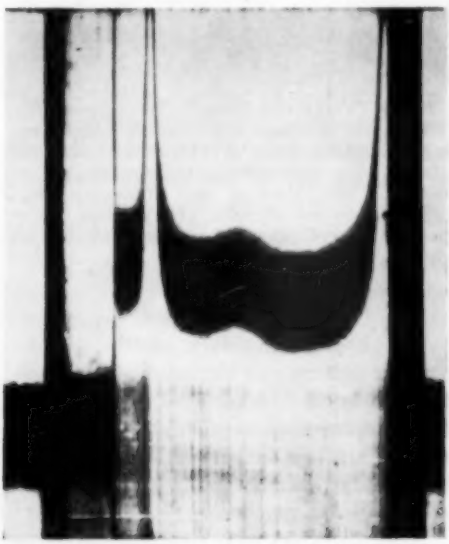

Fig. 6 (Varga and Balazs). Ultracentrifugal pattern of vitreous dissolved from the dried state. Exposure made after 91 minutes. Bar angle: 50 degrees, sedimentation from left to right.

The vitreous, having been liquefied with a syringe, was filtered through an asbestos filter (Seitz K7)* with suction. The filtrate was concentrated six times and dialyzed against pH 7.5 phosphate buffer. In the course of the ultracentrifugal analysis only one boundary was observed. The shape and the sedimentation constant of this boundary were very similar to those of the slow

* Republic Seitz Filter Corporation, Newark, New Jersey.

TABLE 1

SEDIMENTATION CONSTANTS OF VITREOUS AND VITREOUS COMPONENTS

	Concentration (gm./100 ml.)	Sedimentation Constants in S		
		Boundaries		
		1	2	3
Serum albumin	0.2	—	4.60	—
Serum γ-globulin	0.2	—	—	6.50
Hyaluronic acid	0.15	1.60	—	—
Albumin, γ-globulin and hyaluronic acid	—*	1.35	4.55	6.75
Vitreous		0.95	4.10	—
Vitreous and albumin	0.2	1.30	4.24	—
Vitreous and γ-globulin	0.2	2.01		6.50
Vitreous and hyaluronic acid	0.1	1.64	not measured	—

All the ultracentrifugal analyses were made in a 0.006 M phosphate buffer with NaCl, μ 0.12, pH 7.5. The vitreous in all these experiments was five-times concentrated.
* Albumin 0.2, γ-globulin 0.2, Hyaluronic acid 0.1 gm./100 ml.

sedimenting boundary of the unfiltered vitreous preparations. A sharpened leading edge was observed (fig. 10) which occasionally showed up in other experiments, too.

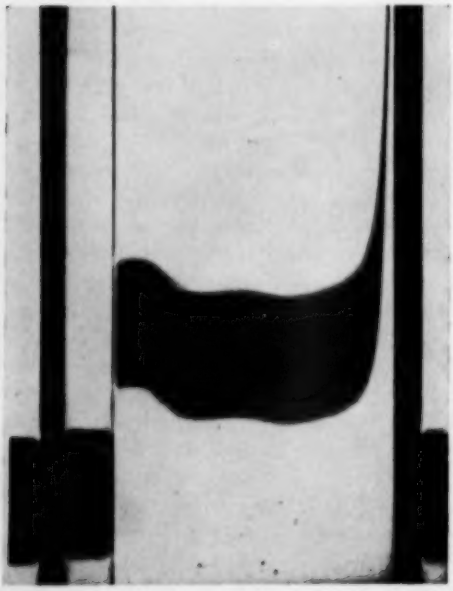

Fig. 8 (Varga and Balazs). Ultracentrifugal pattern of vitreous treated with hyaluronidase. Exposure made after 110 minutes. Bar angle: 50 degrees, sedimentation from left to right.

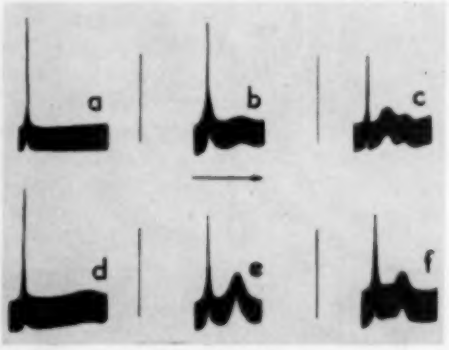

Fig. 9 (Varga and Balazs). The sedimentation patterns of vitreous and vitreous components. (a) Hyaluronic acid. (b) Hyaluronic acid and albumin. (c) Hyaluronic acid, albumin, and γ-globulin. (d) Vitreous and hyaluronic acid. (e) Vitreous and albumin. (f) Vitreous and γ-globulin. Concentration and sedimentation data are given in Table 1.

DISCUSSION

The leading boundary of the electrophoretic pattern was identified by Hesselvik and Blix as the "hyalomucoid" or hyaluronic acid component of the vitreous. In ultracentrifugal analysis the slower boundary could be identified as the hyaluronic acid component of the vitreous.

After digestion with testis hyaluronidase this peak disappeared, and a new boundary appeared with a slower sedimentation value and a tendency to diffuse rapidly. The new

boundary may represent the digested hyaluronic acid or a protein residue, liberated during digestion, from a hyaluronic acid protein complex.

The sedimentation constants of the hyaluronic acid boundary vary markedly with the concentration. In Table 2 the sedimentation constant of the slow moving boundary decreases to a third of its initial value with a six-fold increase in concentration. The relationship between the hyaluronic acid concentration of the vitreous and the sedimentation constant, or its reciprocal, is not linear. This is in accord with the finding of Ogston and Stanier in synovial fluid. They described the hyaluronic acid boundary of synovial fluid as a sharp, slowly diffusing, slowly sedimenting peak, which is very similar to the hyaluronic acid boundary of the vitreous body.[8]

The lack of a linear relationship between the sedimentation constant, or its reciprocal, and the concentration may be the result of a strong interaction among the particles. Extrapolation to zero concentration in this case would be unjustified.

On the other hand, in view of the possibility of gel formation under the influence of hydrostatic pressure in the ultracentrifuge, the hyaluronic acid peak may represent two things, namely, the boundary of the gel and the boundary of the sedimenting component. In such a case the other components would be sedimenting through a gelified medium and small deviations from the ordinary sedimentation rate could be explained by that fact.

It is an interesting observation, that upon filtering the liquefied vitreous through an asbestos filter (Seitz K7) in a subsequent analysis, only the hyaluronic acid peak can be seen. The sharpened leading edge may indicate the gel-forming character of the substance (fig. 10).

TABLE 2

SEDIMENTATION CONSTANTS OF THE SLOW-MOVING BOUNDARY IN THE VITREOUS

Vitreous Concentration	S
not concentrated	2.45
3.0× concentrated	1.18
4.5× concentrated	1.00
5.0× concentrated	0.95
6.0× concentrated	0.85

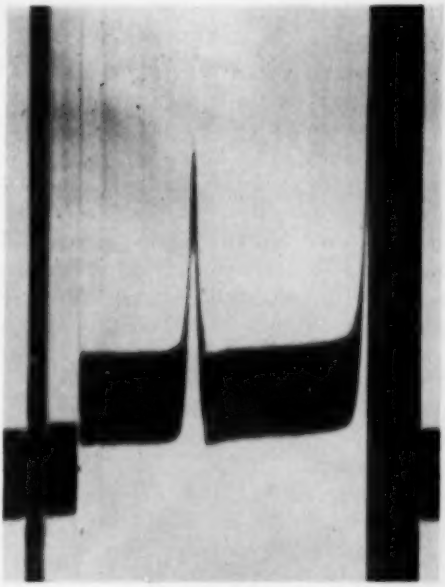

Fig. 10 (Varga and Balazs). Ultracentrifugal pattern of vitreous filtrate. Exposure made after 120 minutes. Bar angle: 45 degrees, sedimentation from left to right.

Hyaluronic acid seems to play an essential part in the space network formation of the vitreous gel. Under the experimental conditions its molecular state is certainly modified. The breakdown of the gel structure during the preparation, caused by mechanical and oxidative factors, may result in some depolymerization of the hyaluronic acid molecules. Nevertheless, there are indications of gel formation under the influence of hydrostatic pressure in the ultracentrifuge.

The sedimentation rate of the hyaluronic-acid boundary was increased in each experiment when albumin or globulin was added to the vitreous. A possible explanation for this observation is molecular interaction be-

tween the hyaluronic acid and the added components.

The leading boundary of the ultracentrifugal pattern had a sedimentation value similar to that of serum albumin. The addition of serum albumin did not change the sedimentation constant. After trypsin and papain digestion this peak completely disappeared, while hyaluronidase digestion had no effect. This observation supports the assumption that this boundary represents the albumin component of the vitreous. In the electrophoretic analysis mobility values of the second boundary were similar to those of serum albumin over the same pH range (fig. 2). This is in agreement with Hesselvik's finding.

The presence of globulins in the vitreous is suggested by the third and fourth boundaries in the electrophoretic pattern. The mobility values of the third boundary are similar to those of plasma γ-globulin (fig. 2). These components have isoelectric points between 6.5 and 6.7, similar to that of bovine γ-globulin, which has an isoelectric point of 6.5 at 0.1 ionic strength.[9] However, from ultracentrifugal analysis there was no indication of a globulin component. Bovine γ-globulin added to the vitreous before ultracentrifugation sedimented at a much more rapid rate than the leading boundary of the vitreous.

Chemical analyses based on the salting-out method clearly showed the presence of a globulin component in the vitreous. Duke-Elder found 11.5 mg. percent globulin and 7.7 mg. percent albumin in the horse vitreous.[10] Cahn[11] reported 44 mg. percent globulin and 33 mg. percent albumin in bovine vitreous body. Using spectrophotometry one of us[6] found 14.5 mg. percent albumin and 31.7 mg. percent globulin in the central sample of the cattle vitreous.

Thus, the fact that ultracentrifugal analysis showed no typical globulin boundary cannot be taken to prove the absence of globulins from the vitreous. The ultracentrifugal experiment on the vitreous with added serum γ-globulin suggests one of two things: either that the globulins, if present in the vitreous, are not γ-globulin type; or that if a γ-globulin is present in the vitreous, there is a molecular interaction and complex formation between the globulin and other components of the vitreous.

Summary

The components of the cattle vitreous body have been studied ultracentrifugally and electrophoretically. A hyaluronic acid and a protein component have been identified by means of treatment with specific enzymes and by addition of reference substances. The results are discussed from the point of view of protein-hyaluronic acid interaction.

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We are grateful for the excellent technical assistance of Miss Elizabeth Anne Eckl.

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GENERAL DISCUSSION ON THE VITREOUS

CHARLES L. SCHEPENS, M.D., *Moderator*

DR. LUDWIG VON SALLMANN (New York): I would like to address my first question to Dr. Schepens. He mentioned the difficulties in the differential diagnosis of membranes in the vitreous from the hyaloid membrane in posterior vitreous detachment. That is an old problem and I am sure Dr. Schepens could have told us more about the differential diagnosis if he had had the time.

I would like to ask him whether he made any attempt to dissect suitable eyes and to demonstrate the hyaloid membrane by means of the pyridine method of Redslob. This would seem to me to be the anatomic proof for the correctness of a slit-lamp diagnosis.

Another question to Dr. Schepens: The holes which he apparently found in a considerable number of cases were caused by the traction of strands that were anchored at the macular region. Does not Dr. Schepens feel that this mechanism is a rather rare one and that most of the macular holes which lead to retinal detachment are of degenerative nature?

DR. SCHEPENS (Boston): As for the first question Dr. von Sallmann asked me, perhaps I did not make it clear that the scope of my paper is purely clinical; the method described by Dr. von Sallmann cannot be used clinically. However, in my opinion, it is not known whether or not some of the pathologic membranes in human eyes, which are not the hyaloid and yet are clearly visible, can be detected by the pyridine method.

As for Dr. von Sallmann's second question concerning macular holes, I did not realize I had conveyed the impression that I had seen a great number of them. I have always been impressed by the fact that the great majority of so-called macular holes are definitely not holes but macular cysts. Whether the majority of macular holes seen clinically are due to degeneration or to traction, I cannot establish at this time.

DR. MARTIN BODIAN (Brooklyn): There are several questions I would like to ask Dr. Schepens, purely from a clinical standpoint.

What is the origin and composition of the ordinary vitreous floater that all of us clinicians see in our offices so frequently? We know there are no serious consequences in most cases, but we would like to know where they come from.

Secondly, what can be done for them? Do lipotropic substances have any real therapeutic value in the treatment of these floaters?

Lastly, do these represent a tendency to retinal detachment, or any other future pathologic condition in the eye?

DR. SCHEPENS: I have not personally carried out any research on the origin and composition of the vitreous floaters, so I can only make a guess and quote from the literature.

It is generally being held that vitreous floaters are rich in proteins; they may be acellular or cellular. When acellular, they are either a condensation of proteins which are normally present in the vitreous body or else they are formed by proteins that were introduced into the vitreous as a result of an inflammation or hemorrhage in the vitreous cavity.

When the vitreous floaters are cellular, they may be either pieces of tissue that have been torn from the tissues that line the vitreous cavity (such as peripapillar tissue in the case of an annular opacity in front of the disc), or they may be pathologic tissue, such as glial or connective proliferation; the latter may result, for instance, from organization of a blood clot or of exudates.

As to what can be done for vitreous floaters, I do not know the answer. Personally, I do not prescribe any treatment for them.

Do they represent a tendency to retinal detachment? I think they represent a definite pathologic process in the vitreous body. Unfortunately we do not know as yet, what is the exact relationship between a pathologic condition in the vitreous body and retinal detachment. We only know that retinal detachment is often due to traction by the vitreous on some part of the retina. Whenever the presence of vitreous opacities is associated with a pull by the vitreous upon the retina, whether the actual mechanism is either shrinkage of the vitreous or simple action of gravity, there is a definite danger of retinal detachment.

DR. ZACHARIAS DISCHE (New York): In our laboratory there is some work being done on the chemical constituents of the vitreous, and I was particularly interested in the work of Dr. Varga and Dr. Balazs because our results in some respects are different from the results they have reported, and my particular interest was in finding out why these differences appear.

First, by which method were these constituents determined, particularly the fibrous structural protein and the hyaluronic acid? That question is directed to Dr. Varga.

Secondly, I think it has been known for quite some time that the hyaluronic acid in the vitreous is present in a polydispersed form, and I wonder how the peak in the sedimentation curve can be explained if we consider that, undoubtedly, a great part of the hyaluronic acid has a much smaller molecular weight than another part in the vitreous.

DR. L. VARGA (Boston): I would like to answer only one of the questions, and I will leave the answer to the other question to Dr. Balazs.

I would like to explain the sharp peak obtained in the centrifuge. It is possible that the hyaluronic acid is an even larger molecule than the protein, but it is a general principle in the ultracentrifuge that, if a component is very highly hydrated it will sediment much more slowly, and the sedimentation is also influenced by the so-called partial specific volume, and, because of these two factors, it can easily be explained why this component is sedimenting so slowly. Naturally, if the component is smaller, it will sediment more slowly, too.

In addition to this, this sharp peak may represent two things: first, it may represent the hyaluronic acid or a hyaluronic acid protein complex, and, in addition to this, the gel and liquid boundary. Because of the shape of the sedimentation constant curve, we drew the conclusion that gelification occurred during centrifugation. These circumstances would explain the nature and sharpness of this peak.

DR. BALAZS (Boston): I would like to answer the question about the sedimentation properties of hyaluronic acid. Sylven and Snellman analyzed a low molecular weight preparation from the umbilical cord and found that the sedimentation constant was 2.7 S.

Ogston and Stanier studied the synovial fluid hyaluronic acid and reported that the sedimentation constant varies with the concentration. Extrapolation to zero gave a limiting value of 1.3 S.

In our experiments, the hyaluronic acid boundary of the vitreous would give a sedimentation constant at zero concentration 2.8 S. However, the validity of the extrapolation is questionable, because of the nonlinear increase of the sedimentation constant at low concentration.

As to the question of polydispersity of the hyaluronic acid, our present experiments give us very little information.

DR. PAUL MILES (St. Louis): In a teaching institution we sometimes see vitreous where it should not be. For instance, during cataract surgery one may find vitreous following the lens up into the anterior chamber. We are looking for something we can use to dissolve the vitreous.

Following healing from a cataract operation there may be vitreous in the anterior chamber and up against the endothelium, which is a serious complication and frequently results in loss of the eye.

In one case we injected Wydase full strength into the anterior chamber two weeks after cataract operation, with very excellent results. I wonder if there may be a better substance than Wydase for dissolving vitreous—a substance which is not harmful to certain structures in the eye.

DR. SCHEPENS: For the benefit of some of the research workers who are not familiar with it, what is Wydase?

DR. MILES: It is Wyeth's brand of hyaluronidase. It is an enzyme.

DR. SCHEPENS: I don't think basic research on the vitreous body has yet reached the point where Dr. Miles' question can be answered adequately. Most of this research work has been done on cattle eyes, some on human eyes. We are still struggling to determine what are the normal constituents of the vitreous body. We haven't even reached the stage where we think we know something about the real structure of the normal vitreous gel. It is therefore impossible, at present, to suggest a rational therapy for disturbances in the vitreous body. If there is somebody in the audience who has a more adequate answer to Dr. Miles' question, we would like to hear it. Does anyone have a suggestion? I am sorry, Dr. Miles; your question remains unanswered.

DR. DAVID COGAN (Boston): I would like to ask Dr. Balazs a question in connection with the absorption curve that he assumes to be due to ascorbic acid in the vitreous. How would he answer the possible objection that it was nucleoprotein that diffused into the vitreous from the retina, rather than the ascorbic acid that he was testing, since they both have an absorption peak around 265?

DR. BALAZS: The nucleoproteins and nucleic acids are undialyzable. The absorbing substances with a maximum at 265 mμ is dialyzable in vitreous. The absorption band of purines and pyrimidines, responsible for the absorption band of nucleic acids, is located at 258 to 260 mμ in pH range of 3.0 to 6.0.

DR. HARVEY THORPE (Pittsburgh): Dr. Schepens, I wonder whether you would tell us more about the membranes that you projected in the slitlamp drawings, the ones that were in front of what you indicated to be the posterior membrane.

Would you care to tell us whether you think they are an intensification of previously existing anatomic membranes, so-called "membranous structures in the vitreous," or whether they are new formations as a result of precipitation of substances in the vitreous?

DR. SCHEPENS: Unfortunately, I know nothing certain about the origin of equatorial membranes. The first point to determine, I think, is whether such membranes actually originate in the equatorial plane of the eye or whether they have migrated, for instance from the vicinity of the posterior pole, to the equatorial plane. It is conceivable that, in some cases at least, such a membrane is really the hyaloid, which has gradually shrunken away from the retina, until it occupies the equatorial plane of the globe.

DR. FREDERICK H. VERHOEFF (Boston): I should like Dr. Schepens, or somebody, to discuss the question of Moore's lightning streaks. A good many years ago, much to my surprise, I found they were dependent upon motion of the eyes. It was obvious that just the pulling of the muscles on the sclera could not account for them, so the only other explanation I could think of was that they were due to shrinkage of the vitreous, and that the vitreous was either pulling or pressing on the retina. It seemed to me that the clinical observations indicated that it was pressure of the vitreous on the retina.

You would think, then, that in these cases there would be frequent separation of the retina, according to the idea that shrunken vitreous does pull on the retina, but, to the contrary, lightning streaks seem to be a good omen. In other words, if you get lightning streaks you can feel pretty sure you will not have separation of the retina, which is a consolation to me, because I have them in both eyes.

It occurred to me that the explanation might be this, and I would like to know whether this would agree with your ideas: If shrinkage of the vitreous is going to cause separation of the retina, it will do so early, and the patient will come in complaining of symptoms due to separation of the retina. If the patient comes in complaining only of lightning streaks, he has passed the danger period, and it is a good sign that he will never get separation of the retina.

I haven't heard you discuss the question of lightning streaks. I would like to ask you to do so.

DR. SCHEPENS: There wasn't time enough in 20 minutes, Dr. Verhoeff, to discuss that question, but what little I know of it is in agreement with what you say. First of all, it is my impression that lightning streaks occur predominantly on the temporal side. Is that true, Dr. Verhoeff?

DR. VERHOEFF: From my own experience, they may occur everywhere. At first, by moving my eye in certain ways, I could get a complete circle. Then, after a few days, I could get only the temporal streak. The only thing most people ever note is the temporal streak.

DR. SCHEPENS: Right; and my explanation for its being temporal is that the nasal periphery of the retina is more sensitive to light than the temporal periphery. When the deep portion of the vitreous body is examined, in a patient complaining of lightning streaks, a vitreous detachment is generally found. Small retinal hemorrhages and exudates are often found in the fundus periphery along a line which probably corresponds to the attachment of the vitreous membrane to the retina.

It has been my experience that lightning streaks are relatively frequent in people who get retinal detachment. However, it is true that the majority of those who have lightning streaks do not get detachment.

DR. VERHOEFF: Moore never saw a case, nor did I, of separation of the retina in a patient who came in complaining of lightning streaks.

DR. SCHEPENS: There are a number of such cases on record, unfortunately.

DR. H. F. ALLEN (Boston): I have a question which might be of some practical import, and which also might be fairly asked of a basic-science symposium such as this:

What is known about the reversibility of the gel-like state, the fluid gel interchange, in the vitreous? We tend to think of fluid vitreous as being a pathologic stage clinically. We have heard of methods of liquefying the vitreous here. Is there any reversibility to this in the laboratory, or any method in use?

DR. VARGA: I mentioned at the very beginning of my lecture that, after the liquefaction of the vitreous it will not regelify again by itself. The only thing I can say is that there seems to be some reason to believe that regelification could occur in the ultracentrifuge under the influence of hydrostatic pressure; however, this has still to be proved by more experiment and facts.

There are certainly such kind of gels which are reversible. The vitreous, according to our experiments, is not reversible, and I am sorry I cannot answer more at the present time and tell you what would reverse the situation.

DR. BRITTAIN F. PAYNE (New York): On behalf of the society we want to thank Dr. Schepens for his masterful handling of this intricate subject. I must say that I was lost most of the time, but found it very interesting when Dr. Verhoeff, asked something of practical importance about "light flashes" which sometimes affects one of my eyes.

I had the distinct privilege of having Dr. Schepens as a guest of the New York Academy of Medicine when he first came to this country. As a matter of fact, I think he directed his first retinal detachment operation on a patient on my service at the New York Eye and Ear Infirmary. He directed Dr. Krug, a member of the staff here and also at the New York Eye and Ear Infirmary, in the reattachment of that retina.

I want to report that the patient did exceptionally well, Dr. Schepens, and, unless I have been misinformed, she still has useful sight.

There is one question I would like to have clarified before we close this symposium and take a short recess. We, who have been interested in teaching microscopic anatomy of the eye for some time have often explained to our students that vitreous opacities probably arise from the ora serrata due to the attachment of the vitreous and also the retina in this region. I am not quite sure that we were prepared to go as far at any time, up to now, and say that there was actually a shrinkage and detachment of the vitreous, because I don't think we quite appreciated that from the specimens.

I wonder if Dr. Schepens would care to comment on this attachment and the strain which is put on the pigment epithelium in the region of ora serrata by these factors—first, the so-called base of the

vitreous; second, the union of the retina in this particular region.

We know that cystic degeneration of the retina occurs mostly on the temporal side, and it seems to be a physiologic process—at least we say so—and I wonder if that doesn't cause the development of these vitreous changes.

In other words, we used to call the condition a type of quiet choroiditis in this region, you might say, which causes a separation of the cellular elements, as well as degeneration of the pigment epithelial cells, and not necessarily always the result of hemorrhage.

DR. SCHEPENS: I don't think I can answer this question adequately as it is a very broad and difficult one. The role played by alterations near the base of the vitreous, in the production of vitreous opacities, is not clearly understood, mainly because it is difficult to examine this area in the living. Of course, there are many cases showing degeneration or a moderate degree of inflammation in this region, but is this related to the vitreous opacity? If it is, which comes first?

If vitreous opacities originate in the extreme fundus periphery, they by no means always have that origin. In highly myopic patients for instance, changes around the posterior pole are probably the cause of centrally located vitreous opacities.

In case of vitreous detachment without visible pathology in the periphery, an annular vitreous opacity is often present.

Another type of case is that of the highly myopic eye showing an annular opacity, but no detachment of the hyaloid membrane from the retina. Such an opacity is probably the result of traction by the vitreous on the area of the optic disc; this causes an annular piece of peripapillar tissue to be torn off, without actual detachment of the hyaloid membrane.

Vitreous opacities may thus result first from changes in the extreme periphery; second, from detachment of the hyaloid; third, from changes in the vitreous gel.

Other mechanisms may cause vitreous opacities, but there is so little evidence to substantiate these hypotheses that I apologize for taking up your time to comment on things I know so very little about.

STUDIES ON THE CORNEA*

I. THE FINE STRUCTURE OF THE RAT CORNEA

MARIE A. JAKUS, PH.D.
Boston, Massachusetts

Reviews of histologic studies of the cornea may be found in standard textbooks and handbooks of ophthalmology. Among the latter, one of the most useful was found to be the volume edited by Möllendorff,[1] which was published during the period in which the electron microscope was going through the stage of its development just preceding its use as a research instrument. Since the late 1930's, the number of published papers dealing with the fine structure of both biologic and other materials has increased greatly; apparently, however, little use has been made of the electron microscope in studying the cornea.

In 1942, Schmitt, Hall, and Jakus[2] examined unfixed beef corneal stroma and found long fibrils of relatively constant width (about 500A†) embedded in a tough matrix which made it difficult to tease out the fibrils and obscured their periodic cross banding.

Wolpers,[3] in 1944, fixed corneas in one-percent OsO_4, cut them with a freezing microtome, and exposed the sections to high frequency sound waves. From beef cornea he obtained typically cross-striated collagen fibrils about 200 A in width. Van den Hooff,[4] in 1952, also examined collagen from fragmented fixed and unfixed beef corneas. He obtained fibrils having widths between 350 and 400 A from OsO_4-fixed corneas, and between 450 and 500 A from unfixed cor-

* From the Retina Foundation, Massachusetts Eye and Ear Infirmary, and the Department of Ophthalmology, Harvard Medical School. This investigation was supported by grants from the American Optical Company; PHS B-56R and B-56C from the National Institute of Neurological Diseases and Blindness, of the National Institutes of Health, Public Health Service; and the Vivian B. Allen Foundation, Inc.

† A = Ångström unit = 10^{-8} cm.

neas. Both had the normal periodicity for collagen. He considered the nonfibrous material adhering to the collagen to be adsorbed ground substance.

In 1953, Schwarz[5, 6] investigated collagen fibrils from both fixed and unfixed human corneas, using the method of Wolpers to fragment the tissue. He observed fibrils which, when freed of their coating of "cement substance," had an average width of 290 A and an average period of 600 A. He was able to remove the adsorbed material from the fibrils by prolonged treatment with sound waves or by digestion with hyaluronidase. Sebruyns,[7] in 1950, examined the cornea by a double-replica method. His micrographs, however, show structures so different from those to be described here that a comparison would be difficult.

Many investigators have studied the cornea in polarized light. Von Ebner, back in 1899 (cited by Lauber[15]), noted that the cells of the corneal epithelium were doubly refractile. For the squamous cells, he calculated an index ellipse which was parallel to the plane of the epithelium; for the basal cells it was perpendicular, and smaller in magnitude. In each case, however, the birefringence was positive with respect to the long axis of the respective cells.

More recently Naylor[8] reported the results of a polarized light analysis of cat corneal stroma. He found both form and intrinsic birefringence and, on the basis of his results, postulated a structure not inconsistent with the observations reported here.

Another approach followed in studying the eye has been that of histochemistry. The results of an intensive study of the anterior segment of the rhesus monkey eye were published by Wislocki[9] in 1952. Some of his findings will be cited, with the implied reservation that his observations may not be extrapolated to the eye of the rat.

The present investigation was undertaken in order to work out a satisfactory technique for sectioning eye tissues. The rat was chosen because it is readily available and has a thin cornea (less than 0.2-mm. thick), which is an advantage when using slowly penetrating fixatives.

Methods

Corneas were excised from rat eyes immediately after the animals had been killed, by ether or by decapitation, and fixed in buffered osmium tetroxide (one-percent OsO_4 in veronal acetate buffer, after the method of Palade,[10] or in veronal carbonate buffer, final pH 7.3 to 7.5) for periods varying from one hour to six hours. They were then washed in several changes of the respective buffer or in distilled water, and dehydrated in the conventional manner. While in 95-percent alcohol, the corneas were trimmed and the central portions were cut into strips approximately 0.3 mm. by 1.0 mm. in size. If it was desired to increase the contrast in the tissue, phospho-12-tungstic acid was added to the last change of absolute alcohol, in a concentration of 0.1 percent.

After dehydration, the strips of cornea were transferred to a 1:1 mixture of absolute alcohol—methacrylate monomer* (five- to 10-percent methyl methacrylate in n-butyl methacrylate), and then into the pure monomer. The tissue was left overnight in the last of several changes of the monomer to insure penetration and was then put into monomer to which had been added a catalyst, Luperco CDB,† in a concentration of 20 to 35 mg./ml. Finally, each strip of cornea was oriented in a gelatin capsule filled with the catalyzed monomer, and the plastic was made to polymerize by placing the filled capsules in an oven at 45°C. or under an ultraviolet lamp for about 12 hours.

Sections were cut with a glass knife[11] in an International Minot rotary microtome adapted for thin sectioning[12] and floated on the surface of an aqueous solution of acetone or dioxane, or on distilled water, held in a

* Rohm & Haas Company, Philadelphia 5, Pennsylvania.

† Lucidol Division, Novadel-Agene Corporation, Buffalo 5, New York.

trough attached to the knife. They were picked up on conventional copper specimen grids covered with thin supporting films. Suitably thin sections were selected on the basis of the interference colors they showed as they floated on the liquid surface.

The sections were usually examined directly, that is, without removing the plastic embedding material. The electron microscope used was an RCA Model EMU-2D, equipped with condenser and objective apertures. Most photographs were taken at relatively low magnifications (×2,000 to ×6,000), in order to cover wider fields, and enlarged subsequently.

Observations

Epithelium

The nucleus of the *basal cell* (figs. 1, and 2) is roughly spherical in shape but it may be more or less lobed, with the indentations sometimes extending inward deeply. In these OsO_4-fixed cells it appears granular and is of relatively uniform density, except when the section includes nucleolar material. Nucleoli are denser than the surrounding nucleus and have the usual wormlike shape. In the cells of the cornea they are not prominent.

Around the nucleus are many granules of varying size and density. Among these are the mitochondria, which appear dark in micrographs and which show characteristic internal structure.[13] The mitochondria vary in size and shape, due in part to the different planes in which they were cut, and measure 0.1 to 0.2 μ in width and up to 1.0 μ in length. Another type of granule is also found with some frequency. They are denser than the surrounding cytoplasm but less dense than the mitochondria and may be oriented linearly or in small clusters. In diameter they are about 900 A.

Filling the spaces between the granules, the cytoplasmic matrix appears as a meshwork of fine filaments with occasional small vesicles along them. These filaments probably correspond to the endoplasmic reticulum described by Porter.[14]

The cell membrane is thin, visible only as a single, or sometimes double, fine line. It may, however, appear thicker and dark when dense material is concentrated in that region. This is seen particularly in the basal portion of the cell, where the adsorbed layer may be a quarter of a micron or more in thickness and may include mitochondria.

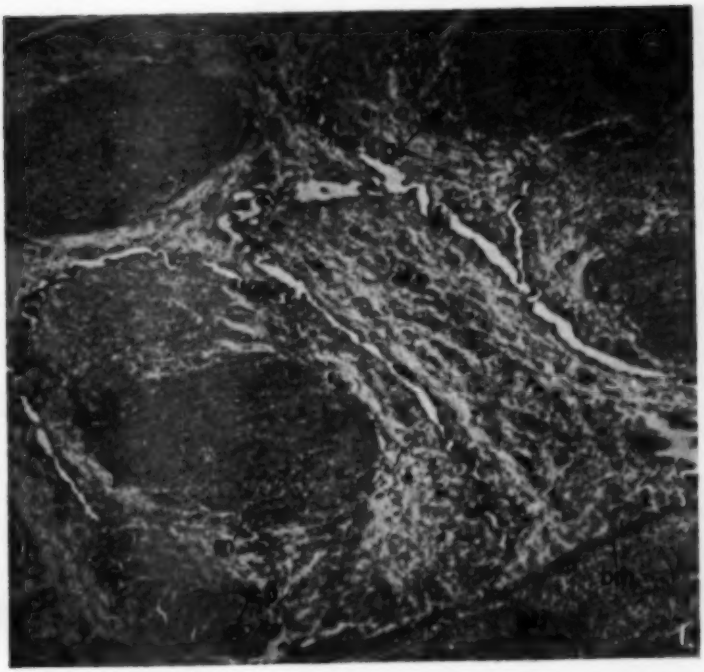
The surfaces of the basal cells are drawn out into many finger-shaped processes which fit into complementary grooves and pits between the processes of the adjacent cells. The extensions near the base of the cell tend to be thicker than those toward the apex, and may, indeed, form broad "feet" on some cells.

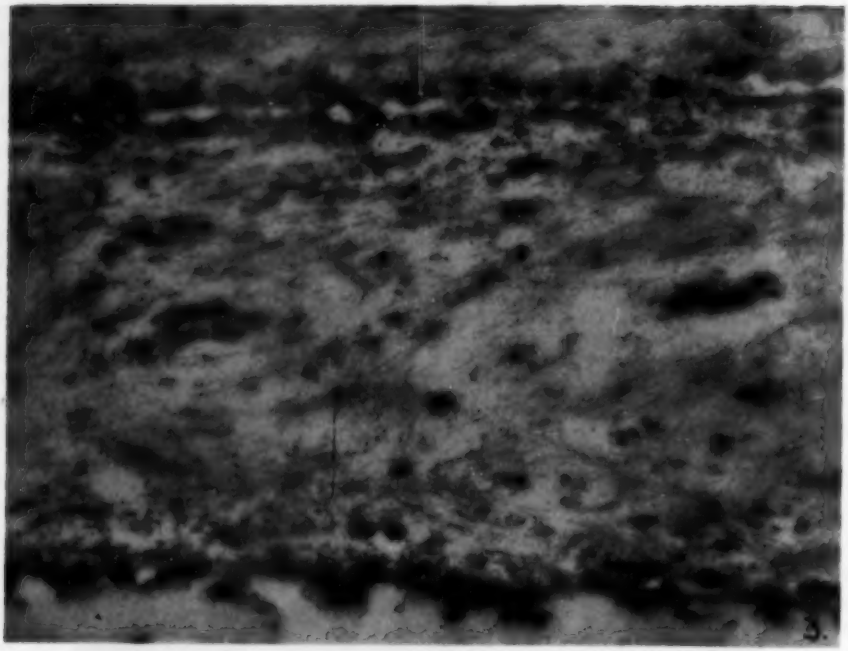
In the *wing cells* (fig. 2), all the structures described in the basal cells can be found but in somewhat moderated form. The nucleus, now elongated in a direction tangential to the cornea, has shallower indentations. The granules and inclusions appear to be fewer in number, thus allowing more of the filamentous matrix to be visible. Even the interdigitating processes are more slender.

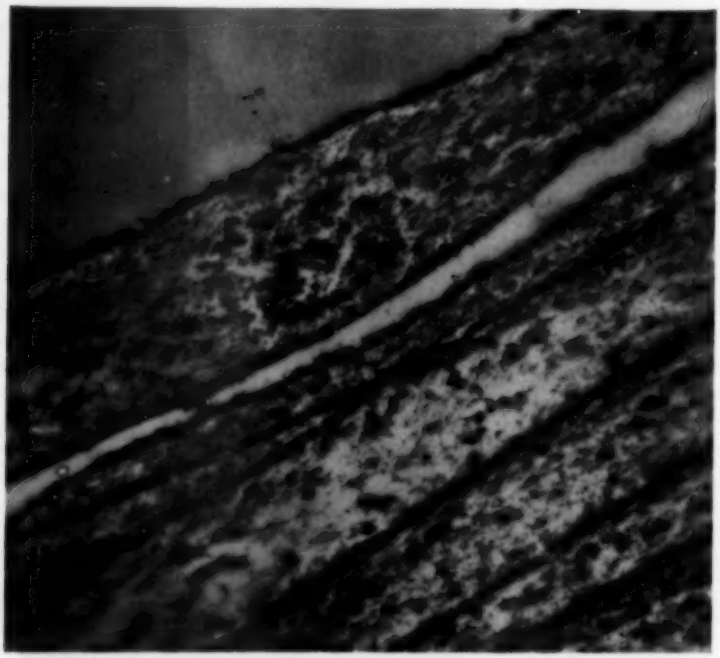
In the *squamous cells* (figs. 2, 3, and 4) these modifications are carried still further. The nuclei are flatter and smoother in contour. The granular inclusions are even more sparsely distributed, as are the cell processes. The only component which has become more prominent is the cytoplasmic filamentous system (fig. 3). These fine filaments run roughly parallel to the cell membranes and to each other, forming bundles which course along between the cell inclusions. In sections of

Fig. 1 (Jakus). Shows two basal cells, one cut through the nucleus, and portions of other basal and wing cells. Structures seen are stroma (str), basement membrane (bm), cell processes (cp), mitochondria (m), and granules (gr). (×4,500.)

Fig. 2 (Jakus). Shows portions of two basal cells, a wing cell, and two squamous cells. The nucleus of the left basal cell has a deep indentation. (×4,450.)







cornea which have been treated with phosphotungstic acid, the filaments stand out more clearly (fig. 4). When suitably oriented, they appear as long threads having rough surfaces and widths up to about 200 A.

Basement membrane

Directly beneath the basal cells of the epithelium, and separating them from the stroma, is the basement membrane (figs. 1, 5, 6, 7 and 10). In fixed corneas, the adhesion of this structure to both the stroma and the epithelium appears to be very strong; when the epithelium tears away from the stroma, the tear is always just above the cell membranes of the basal cells, that is, within the cytoplasm of these cells (fig. 5).

In thicker sections, the basement membrane is a smooth, dense line, about 300 A thick and distinct from the cell membranes of the basal cells (fig. 6). In thinner sections, it appears to be a condensation of fibrils, which are considerably narrower in width than are the collagen fibrils of the stroma (fig. 10).

The rat cornea does not have a Bowman's membrane.

Stroma

The most conspicuous component of the corneal stroma is the collagen. This takes the form of fine fibrils, uniform in diameter and showing a high degree of orientation throughout most of the stroma. They lie in ribbonlike bands running parallel to the surface of the cornea, and within each band they are oriented parallel to each other and to the long axis of the band (figs. 5 and 9). Just under the basement membrane there is a relatively narrow region in which the arrangement of fibrils may be somewhat haphazard (fig. 10).

Channels of varying size are frequently seen between groups of lamellae (fig. 5). They may appear empty, that is, devoid of formed structures, or they may contain granules (fig. 5) or cells. Some of the cells observed undoubtedly are the "corneal corpuscles"; others appear to be Schwann cells (fig. 7) containing C fibers (C fibers are unmyelinated nerve fibers, of small diameter and slow rate of conduction, which are thought to carry pain impulses). If, during fixation, the cornea shrinks or becomes distorted, many of the channels disappear and the orientation of the lamellae becomes distorted (fig. 11).

In order to determine the diameters of the collagen fibrils, several hundred measurements were made from enlarged micrographs of phosphotungstic acid-stained stroma sections. Most of the diameters fell between 200 and 300 A. There was, however, a discrepancy between the positions of the peaks of the distribution curves (diameter vs. number of fibrils having that diameter) constructed for measurements of (1) cross sections of fibrils and (2) fibrils lying in the plane of the section. For cross sections, the peak of the curve lay between 250 and 300 A, with an average value about 275 A. For fibril segments, both long and short, the peak lay between 200 and 250 A, with the average about 230 A.

Little information could be got from sections about the lengths of the collagen fibrils because of the thinness of the sections and the usual lack of coincidence between the plane of the section and the planes of the fibrils. Corneas were thus fixed in the usual way, washed and fragmented in a Waring

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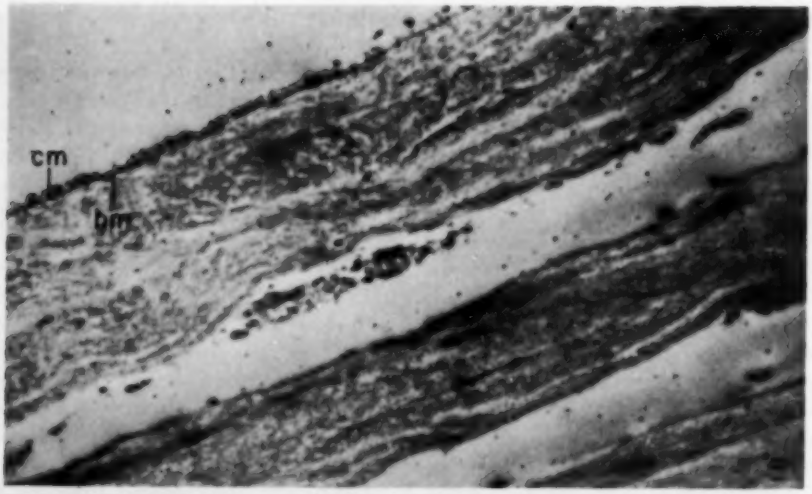
Fig. 3 (Jakus). Shows portions of two squamous cells. The cytoplasm has relatively few granules but many fine filaments. (×19,000.)

Fig. 4 (Jakus). Shows portions of a number of squamous cells. The uppermost cell was sectioned near its nucleus and includes some of the nearby granules. This cornea had been treated with phosphotungstic acid, which increased the density of the cytoplasmic filaments and of the cytoplasm immediately adjacent to the cell membranes. (×7,000.)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8

Blendor. The suspension was centrifuged and washed several times. Specimens were made, stained with phosphotungstic acid and shadowed lightly with chromium to increase the contrast. Fibrils so obtained appeared to be of indefinite length (fig. 12). Relatively few ends were seen and many of these were blunt, suggesting breakage during preparation.

It might be noted that fibrils prepared in this manner appear wider (300 to 400 A) than the fibrils seen in sections. This can be expected, however, since isolated structures in aqueous suspension, when allowed to dry down on a supporting film, always show some degree of flattening. In sections, on the other hand, fibrils cut in cross section appear as solid circles or polygons with rounded angles.

The periodicity of the stroma fibrils, measured both in sections and in isolated fibrils, fell in the range found for other types of collagen, between 625 and 700 A. In favorable sections, fibrils are seen which have several subbands within the major period (fig. 10).

Between the collagen fibrils, in sections of stroma, lies an irregular meshwork of very fine filaments which are sometimes concentrated into cloudlike masses (fig. 10). Around fibrils cut in cross section this, or some other component, often forms an irregular, closely applied coating.

Descemet's membrane

The attachment of Descemet's membrane to the stroma appears to be a very firm one. When separation does occur, the tear almost invariably takes place within the stroma, leaving one or more layers of collagen fibrils adhering to the membrane.

In unstained preparations which seem to be well fixed, the appearance of the bulk of Descemet's membrane justifies the use of the adjective "structureless," even with the high resolution obtained with the electron microscope (fig. 13). Just beneath the stroma, there is a region which may appear fibrous, or laminated, but the higher density of this layer makes it difficult to discern its components.

In sections of corneas which have been treated with phosphotungstic acid, Descemet's membrane is somewhat less homogeneous in appearance. Small granules and very fine filaments can be discerned, as well as larger fibrils. Those near the stroma have thus far been too dense, and surrounded by almost equally dense material, to permit study of their structure. Other fibrils, few in number and appearing usually near the endothelial surface of Descemet's membrane, are cross-striated (the few periods measured have been under 200 A).

If, during fixation or preceding it, the pH or tonicity of the solutions are not favorable, Descemet's membrane swells, at times to a marked degree. Swelling usually begins in the middle two thirds of the layer, which may then become quite fibrous (fig. 14). The fibers are, however, somewhat fuzzy in contour and they may anastomose, forming what look like incompletely separated layers. Although this configuration is seen in most swollen membranes, others have appeared swollen without being fibrous.

Endothelium

In contrast to the strong adhesion to each

Fig. 5 (Jakus). Shows two channels in the stroma. The upper one has within it a few granules and a portion of a cell process. The epithelium has been torn away, leaving the basement membrane (bm), the membranes of basal cells (cm), and some cytoplasmic inclusions. (×5,300.)

Fig. 6 (Jakus). Shows portions of stroma (str), basement membrane (bm), and cell membranes (cm), cytoplasm and mitochondria (m) of basal cells. This cornea had been treated with phosphotungstic acid. (×34,750.)

Fig. 7 (Jakus). Shows in the stroma beneath the basement membrane, a portion of a Schwann cell containing C fibers, with their stalklike mesaxons (ma) and included mitochondria (m). (×12,450.)

Fig. 8 (Jakus). Shows a portion of a phosphotungstic acid-stained Descemet's membrane, at greater magnification. In this region, near the endothelium, appear occasional striated fibrils (f). (×64,150.)

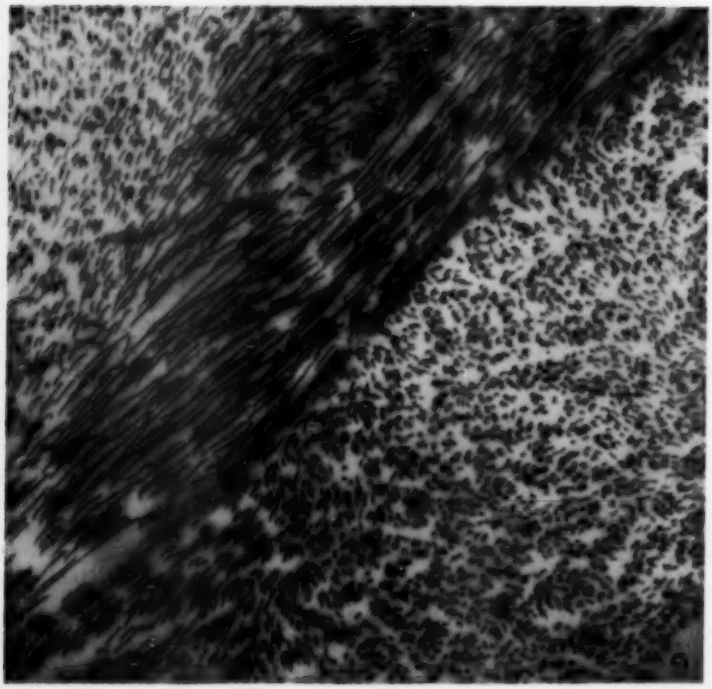
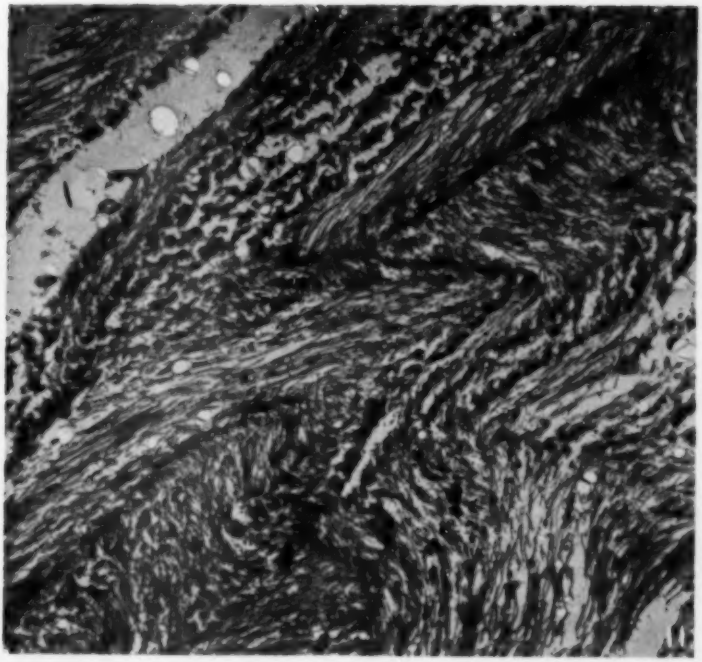


Fig. 9 (Jakus). A section through several lamellae of the stroma, showing the arrangement of the collagen fibrils. Contrast has been increased by phosphotungstic acid. (×15,500.)

Fig. 10 (Jakus). Shows the fibrous nature of the basement membrane (bm), the fine filaments (fil) between the collagen fibrils of the stroma, and the periodicity of the collagen (c), with numerous fine bands in each large period. (×37,200.)



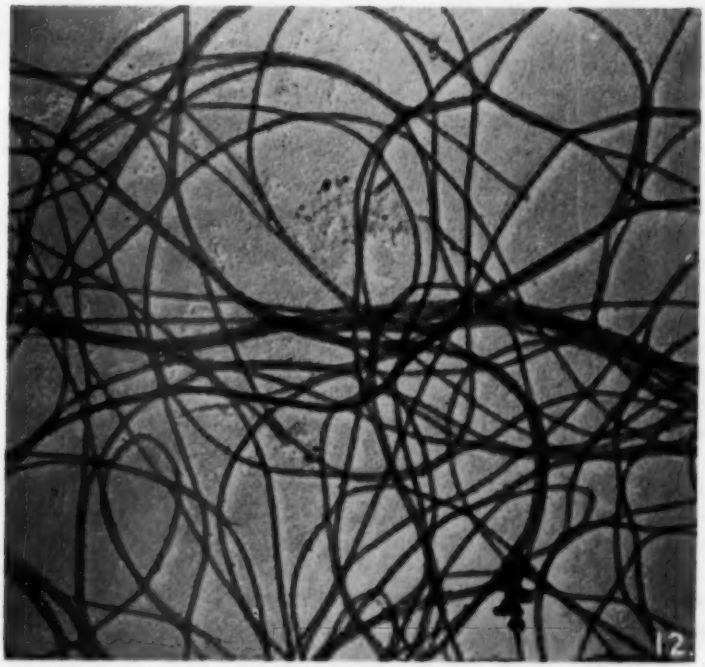


Fig. 11 (Jakus). The plastic embedding medium was dissolved away from this section of stroma, and the specimen was subsequently shadowed lightly with chromium. As a result of distortion during the fixation, most of the open spaces between lamellae are obliterated, and the lamellae themselves have become disoriented. (×4,000.)

Fig. 12 (Jakus). Shows collagen fibrils, isolated from fragmented fixed corneas. Contrast was increased by washing, treatment with phosphotungstic acid, and shadowing. (×20,500.)

other exhibited by the other layers, the endothelium of the rat cornea has shown a great reluctance to remain in place and to remain intact.

Endothelial nuclei are greatly flattened, and similar to the nuclei of squamous epithelial cells (fig. 14). The cytoplasm, however, appears quite different (fig. 13). It contains granules of varying size but not the distinctly filamentous component of the epithelium. The cells are irregular in thickness and delimited by thin membranes.

Discussion

In the preceding section certain observations were made which confirm or amplify observations, or hypotheses, made by other investigators. Other observations seem to be in disagreement with earlier work. However, it must be kept in mind that this study is confined to the cornea of one animal, the rat, and until further studies of a comparative nature are made, the findings reported here should be applied only to rat corneas treated in the manner described.

The filaments seen in the cells of the epithelium appear to be sufficiently numerous to account for the double refraction observed in the epithelium. The orientation, too, is such that it would produce form birefringence of the proper sign. The weaker activity of the basal cells could be the result of both less precise orientation and a somewhat lower relative concentration of filaments, due to a higher concentration of cell inclusions.

Wislocki obtained deep staining of the most superficial squamous cells with the periodic acid-Schiff reagents, and with Pap's alkaline silver nitrate method for reticulum. He interprets the reaction with the periodic acid-Schiff reagents as indicating the presence of a high concentration of protein-linked carbohydrate, probably a mucopolysaccharide. Pap's stain, while giving a strong reaction, resulted in a brown rather than a black color. The suggestion should not be overlooked, however, that the intense staining, particularly with Pap's stain, may be correlated with the presence of a high concentration of a filamentous component in the squamous cells.

In none of the sections studied were intercellular bridges or "tonofibrils" seen. Each cell appeared to be a structural unit, in close contact with adjacent cells, but having no direct connection with them.

The collagen fibrils of the stroma are characterized by relative constancy in diameter and a high degree of orientation within birefringent bandlike lamellae. While having the characteristic master period of collagen, they do not always show many subbands within the larger period. This is particularly true of collagen fibrils isolated from fragmented stroma.

Since repeated washing and prolonged extraction of the stroma with salt solutions, to remove mucopolysaccharides, improve the picture somewhat, it appears that isolated corneal collagen fibrils are coated with an adsorbed layer which obscures their surface structure, as was concluded by Schwarz. Further evidence for the presence of such an adsorbed layer is seen in the halo around many fibrils cut in cross section. This halo, if dense enough, serves to increase the diameter of the fibril, and probably accounts for the larger diameters of fibrils cut in cross section.

It need scarcely be pointed out that the picture obtained from sections of fixed material cannot be extrapolated to give that of the living stroma without modification. Certainly the configuration of collagen fibrils invested with coats of "ground substance" and lying in a matrix empty except for a loose meshwork of filaments would not be compatible with transparency of the cornea. One

Fig. 13 (Jakus). Shows stroma, Descemet's membrane, and portions of endothelial cells. (×5,250.)
Fig. 14 (Jakus). Shows Descemet's membrane, swollen and fibrous. Below is the nucleus of an endothelial cell and fragments of its cytoplasm. (×4,550.)

13

14

can only assume that fixation and dehydration cause a separation and redistribution of the highly-hydrated material which normally fills the spaces between the fibrils and which prevents sharp discontinuities in refractive index.

The widely varying appearance of Descemet's membrane is in accord with the conflicting reports of different investigators cited by Lauber. In most instances the degree of fibrous structure observed seems to bear a direct relationship to the amount of swelling of the membrane. Apparently well-fixed membranes show little well-resolved structure, other than the relatively few fibrils seen adjacent to the stroma and near the endothelium. If these fibrils are collagen, they are still far too few in number to account for the similarity of the staining reaction of Descemet's membrane to that of collagenous tissue observed by Wislocki. Furthermore, the abundant "ground substance" would appear to be different from that of the stroma, since the latter stains metachromatically with toluidin blue while the former does not, according to Wislocki. It can only be said at this time that Descemet's membrane is not made up of typical collagen, and further work is needed to determine its composition.

Conclusions

The fine structure of the cornea of normal rats has been investigated. Thin sections were cut and examined with the electron microscope. Because of the high resolution obtained with this instrument (better than 100 A in suitably thin preparations), details of structure were seen which could not have been resolved with visible light. Among these may be listed the interdigitating processes of the epithelial cells and the filaments in their cytoplasm; the fibrous nature of the basement membrane; the collagen fibrils of the stroma; and the variations in appearance of Descemet's membrane which, even at high resolutions, may seem "structureless."

Sections also provide maps of structures, hence reference points with which further observations—on embryogenesis, or on pathologically or experimentally altered tissues—may be compared.

30 Chambers Street (14).

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Discussion

Dr. K. W. Ascher (Cincinnati): I wonder whether the striation depends in any way on the method of fixation of the preparation?

Dr. Jakus: It apparently does not.

Dr. Ascher: Were there specimens without any fixation which looked the same?

Dr. Jakus: When I was at the Massachusetts Institute of Technology, I looked at collagen fibrils from many different sources, including corneal stroma. At that time the unfixed stroma was simply blended or teased apart with needles, and the striation distances found fell into the usual range of collagen periodicity. The same range is observed in fibrils from cornea which has been fixed and then blended or pulled apart into its component fibrils, or in sections of stroma.

The most frequently observed values of the period may be somewhat greater than those seen in other types of collagen. The peak of the distribution curve may fall near 700 A, but the range appears to be the same regardless of whether or not the tissue is fixed.

Dr. David G. Cogan (Boston): I would like to know if Dr. Jakus has treated any specimens with fat solvents before sectioning, and, if so, I am particularly interested to know what the effect of the fat solvent was on the membrane between the epithelium and the stroma.

Dr. Jakus: The corneas thus far examined have not been treated with fat solvents before fixation. However, during dehydration they are put through alcohols ranging from 50 to 100 percent; thus, anything which would dissolve in ethyl alcohol would be taken out. It would be interesting, however, to extract the tissue with petroleum ether, benzene, or other fat solvents.

THE ROLE OF THE ADRENAL CORTEX AND VITAMIN B_{12} IN DIABETIC RETINOPATHY*

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In recent years, the basic pathology of diabetic retinopathy has been rediscovered and adequately described.[1-3] The association of diabetic retinopathy with Kimmelstiel-Wilson renal lesions has been firmly established.[3, 4] In addition, considerable suggestive evidence has been accumulated in the laboratories of The Wilmer Institute to implicate the adrenal cortex in the pathogenesis of this disease complex.[5]

The evidence for excessive adrenal cortical function in diabetics with retinopathy and Kimmelstiel-Wilson disease is derived from several sources:

Clinically, diabetic retinopathy and Kimmelstiel-Wilson disease first appear or are made worse during pregnancy,[5, 6] infections,[7, 8] or the administration of ACTH,[9] all of which are associated with increased adrenal cortical activity; retinal capillary aneurysms have been observed to appear following ACTH administration;[5] the increased urinary excretion of oxysteroids by patients with diabetic retinopathy[10] is indicative of excessive secretory activity of the zona fasciculata of the adrenal cortex; and improvement has been reported following the decreased adrenal cortical function induced by adrenalectomy,[11] postpartum pituitary necrosis,[12] and testosterone administration.[13]

At autopsy, diabetics with retinopathy and Kimmelstiel-Wilson disease, in contrast to uncomplicated diabetics, have heavier adre-

* From The Wilmer Ophthalmological Institute of The Johns Hopkins Hospital, Baltimore, Maryland.

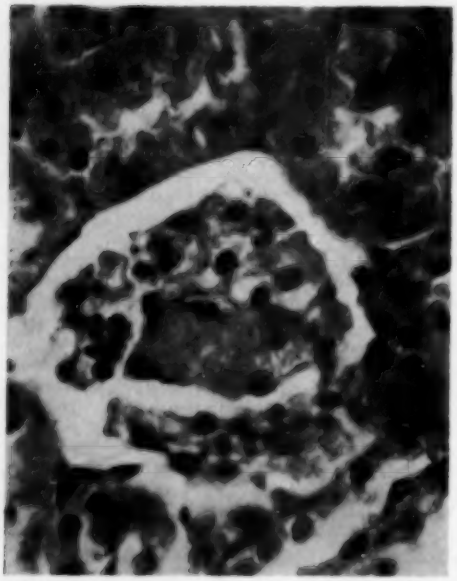

Fig. 1 (Becker, et al.). Cortisone-induced renal lesion in rabbit. Reprinted from the *Annals of Internal Medicine* **37**:273, 1952.)

nals with excessive lipoid vacuolization of the zona fasciculata,[9] and an abnormally high incidence of adrenal cortex adenomas.[14]

Experimentally, furthermore, one can produce glomerular nodules resembling Kimmelstiel-Wilson lesions in rabbits by the administration of cortisone.[15] When ACTH or cortisone is administered to alloxan diabetic rabbits, the incidence of these renal lesions is increased, and retinal capillary aneurysms can be seen[5] (figs. 1 and 2).

Recently, in collaboration with Dr. Bacon F. Chow, the interrelationships of vitamin B_{12}, diabetes and adrenal cortical hormones have been explored.[16] Vitamin B_{12}-deficient animals were reported to exhibit hyperglycemia.[17] Furthermore, administered vitamin B_{12} labelled with cobalt-60 was found in relatively high concentrations in the pancreas, kidneys, and adrenals of experimental rats. Therefore, interest was aroused in the relationship of B_{12} to the diabetic retinopathy—Kimmelstiel-Wilson problem.

Experimental chronic alloxan diabetes in rats was noted to induce B_{12} deficiency, and cause excessive retention of a test dose of this vitamin. Cortisone administration to rats was found to mobilize B_{12} from all tissues and increase its excretion in the urine.[16] This occurred even in the presence of a marked B_{12} deficiency, thus aggravating the deficiency state.[18] The cortisone treated patient also excreted an excessive fraction of a test dose of B_{12}.

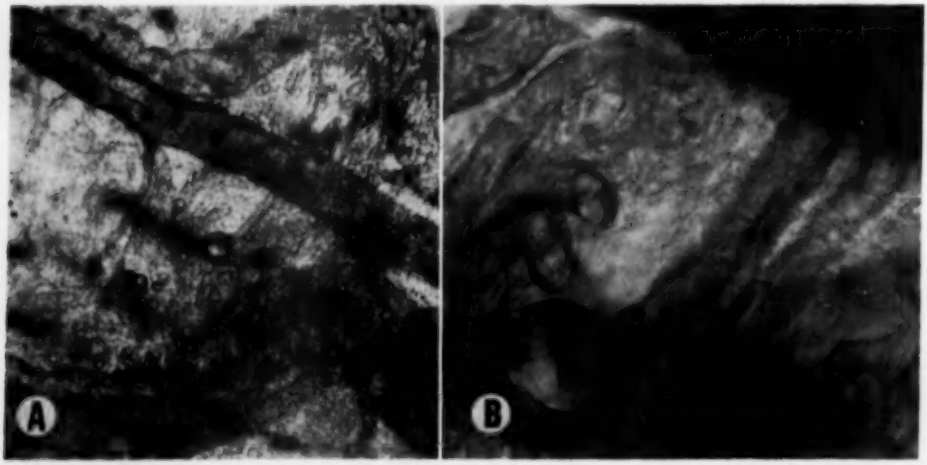

Fig. 2 (Becker, et al.). Capillary aneurysms in flat preparation of retina of diabetic rabbit following ACTH administration. (A) Low power. (B) High power. (Reprinted from the *Annals of Internal Medicine*, **37**:273, 1952.)

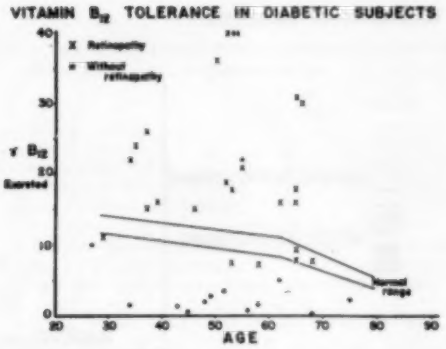

Fig. 3 (Becker, et al.). Urinary excretion following intramuscular injection of 50 μg. vitamin B_{12}. (Reprinted from the *Journal of Clinical Nutrition*, 1:417 [Sept.-Oct.] 1953.)

When an intramuscular test dose or 50 μg. of vitamin B_{12} was given to diabetic patients, and their eight-hour urinary excretion of B_{12} measured microbiologically,[19] striking differences in excretion were noted between the diabetics with and those without retinopathy.[16] Figure 3 represents a scatter diagram of urinary excretion of B_{12} plotted against the age of the patient. There is a significantly greater excretion over the entire age range by the retinopathic than by the uncomplicated diabetic.

Thus the 22 diabetics with retinopathy excreted an average of 19 gamma compared with a normal nondiabetic value of 9.6 gamma. The 13 diabetics without retinopathy excreted an average of only 4.2 gamma, thus retaining more of the test dose than did the normal nondiabetics. Thus the marked difference in the fraction excreted of a test dose of vitamin B_{12} provides a useful clinical test for the presence or absence of retinopathy in the diabetic.

The decreased urinary B_{12} excretion in the uncomplicated diabetic may be interpreted as a B_{12} deficiency, the patient retaining rather than excreting the test dose. If this is true, the diabetic patient is subject to a deficiency of B_{12} as well as of other B vitamins. The increased excretion by the diabetic with retinopathy is more difficult to rationalize. However, it should be noted that such excessive excretion of B_{12} simulates the cortisone treated experimental animal or patient.

These findings are therefore consistent with the thesis of an increased adrenal cortical activity associated with the retinopathic state. The excessive levels of adrenal cortical hormones could prevent the retention of B_{12} even in the presence of a possible B_{12} deficiency in these diabetics. Efforts were made therefore to reduce the endogenous production of adrenal cortical hormones in diabetics with retinopathy in order to reveal their true vitamin B_{12} status.

Testosterone has been reported to decrease adrenal cortical function by inhibiting the production of ACTH by the pituitary.[20-22] When testosterone was administered to diabetics with retinopathy, a marked reduction in the excretion of the test dose of vitamin B_{12} resulted, approaching the nonretinopathic excretion level[16] (Fig. 4). Thus the action of testosterone may be interpreted as having unmasked the B_{12} deficiency of diabetics with retinopathy.

Vitamin B_{12} has been reported to be beneficial in the treatment of diabetic neuropathy.[23] It was therefore of interest to establish the relationship between the neuropathy and retinopathy of diabetes. A review[24] of all cases of diabetic neuropathy in the files of The Johns Hopkins Hospital revealed that over 90 percent of such cases had diabetic retinopathy (table 1). If it can be assumed that B_{12} deficiency plays a role in diabetic neuropathy, this provides additional evidence for the hypothesis that diabetics with retinopathy are even more depleted of vitamin B_{12} than their more fortunate fellow diabetics without retinopathy.

TABLE 1

OCCURRENCE OF DIABETIC RETINOPATHY IN PATIENTS WITH DIABETIC NEUROPATHY

	No. of Patients	Diabetic Retinopathy
Sancetta[23] (1951)	12	10
Becker and Davies[24] (1952)	56*	52

* Only those patients were included whose fundi were examined.

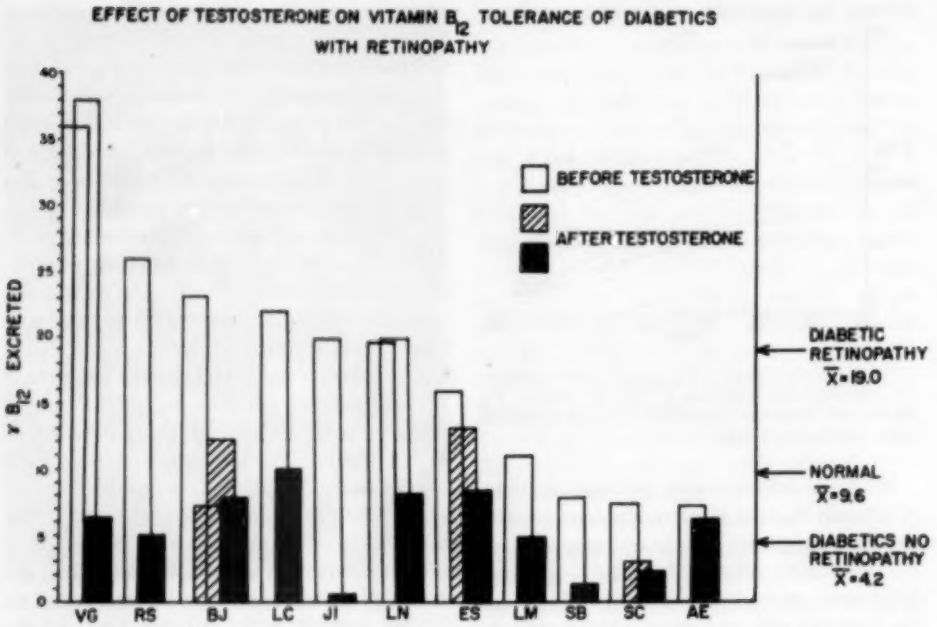

Fig. 4 (Becker, et al.). Effect of testosterone on test-dose excretion of B_{12} in diabetic retinopathy. (Open bars represent B_{12} excretion before testosterone; solid bars are after testosterone administration.) (Reprinted from the *Journal of Clinical Nutrition*, 1:417 [Sept.-Oct.] 1953.)

As pointed out above, alloxan diabetes was found to potentiate the cortisone-induced renal lesion in the rabbit.[14] Since the diabetic state appeared to be associated with a vitamin B_{12} deficiency,[16] the question of the role of such a B_{12} deficiency in the potentiation of the cortisone-induced renal lesion in rabbits seemed worthy of exploration.

Experimental

Albino male rabbits (Research Supply, Philadelphia) weighing four to five lb. were maintained on an experimental diet of Sherwood rabbit pellets* containing no added B_{12} or aureomycin. Control rabbits were maintained on the same diet but containing the usual amounts of added vitamin B_{12} (5.0 mg./ton) and aureomycin (3.6 gm./ton). Other control animals were kept on the unsupplemented diet but given 100 μg. of B_{12} daily (50 μ.g. orally and 50 μ.g. subcutaneously). Cortisone acetate† was administered intramuscularly to all rabbits in doses of 8.3 mg. per day for 21 days. All animals were killed on the 21st day by intravenous pentobarbital and autopsied.

Results

The incidence of renal lesions in rabbits is summarized in Table 2. Lack of a B_{12} supplement increased the occurrence of kidney lesions to 100 percent, and supplementary vitamin B_{12} reduced the incidence to 32 per-

* The experimental diet contained: Protein 19 percent, fat 3.5 percent, fiber 13 percent, calcium 1.0 percent, phosphorus 1.0 percent, salt 1.0 percent, iodine 0.00025 percent, copper 0.00037 percent, manganese 0.012 percent, zinc 0.001 percent, KI 0.0009 percent, ferrous sulfate 0.0088 percent, linseed oil meal, dehydrated alfalfa meal, soybean oil meal, wheat middlings, ground oats and corn, corn gluten feed, irradiated yeast, and vitamin B_{12} supplement.

† Vitamin B_{12} and cortisone were kindly supplied by Merck and Company, Inc., Rahway, New Jersey.

TABLE 2

INCIDENCE OF CORTISONE-RENAL LESIONS IN RABBITS

	No. of Rabbits	Lesions
Control diet[a]	47	15 (32%)
Experimental diet[b]	15	15 (100%)
Experimental diet with oral and parenteral supplement[c]	15	5 (33%)

[a] Regular Sherwood rabbit pellets (5.0 mg./ton B_{12} and 3.5 gm./ton aureomycin).
[b] Experimental pellets without B_{12} and aureomycin.
[c] 50 μg. of B_{12} orally and 50 μg. subcutaneously per day.

cent. There was no significant difference between rabbits that received the small oral supplements and those supplemented with large doses parenterally and orally.

DISCUSSION

The greatly increased incidence of cortisone-induced renal lesions in the rabbit on a diet not supplemented with vitamin B_{12} suggests that the explanation for the observed potentiation of this lesion by alloxan diabetes may be related to an associated B_{12} deficiency. The findings also provide additional evidence for the interpretation suggested for the B_{12} test-dose excretion of diabetics and, in particular, lend weight to the supposition of a marked B_{12} deficiency in the diabetic with retinopathy. At any rate, it would appear that a *B_{12} deficiency* as well as *increased adrenal cortical activity* are involved in the pathogenesis of diabetic retinopathy and the Kimmelstiel-Wilson lesion.

It is entirely possible that both diabetes and adrenal cortical hormones act through a B_{12} depletion mechanism. The lesions might then be due primarily to deficiency of B_{12} and/or an inability to metabolize or methylate some steroids in the absence of B_{12}. In rats, vitamin B_{12} had been shown to counteract almost completely such cortisone effects as thymus atrophy, alopecia and inhibition of body growth.[25]

Deficiency of vitamin B_{12} does not, however, appear to be the sole defect in the pathogenesis of diabetic retinopathy and Kimmelstiel-Wilson lesions. Thus, B_{12} failed to prevent the cortisone-induced renal lesion in all of the rabbits; one third of the animals exhibited lesions in spite of massive doses of B_{12} (table 2). Furthermore, severe deficiencies of vitamin B_{12} in rats do not produce renal lesions resembling those described by Kimmelstiel and Wilson.

It should be noted also that the vitamin B_{12} deficiency present in patients with pernicious anemia is not usually associated with Kimmelstiel-Wilson lesions or diabetic retinopathy.* Finally, intensive vitamin B_{12} therapy failed to alter significantly the clinical course of patients with diabetic retinopathy.[27]

There is ample evidence, therefore, that the retinal and renal lesions of the diabetic cannot be attributed wholly to a vitamin B_{12} dedeficiency. It would seem more likely that adrenal cortical hormones, diabetes, and B_{12} deficiency act together in producing the lesions by means of their effects on some other deficiency or metabolic disorder. Studies are underway to explore such possibilities.

SUMMARY

1. Evidence has been reviewed for implicating excessive adrenal cortical function and vitamin B_{12} deficiency in the pathogenesis of diabetic retinopathy and the Kimmelstiel-Wilson lesion.

2. The vitamin B_{12} test-dose excretion provides a useful clinical test for the presence or absence of retinopathy in the diabetic.

3. The incidence of cortisone-induced renal lesions in rabbits is markedly increased by omission from the diet of supplementary vitamin B_{12} and aureomycin.

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The Johns Hopkins Hospital (5).

* Retinal capillary aneurysms have been described in some pernicious anemia patients.[28] Flat preparations of the retinas revealed microaneurysms in only two out of eight available retinas from cases of *untreated* pernicious anemia in the autopsy files of The Johns Hopkins Hospital.[31]

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Discussion

Dr. von Sallmann (New York): May I ask Dr. Becker about the dose of cortisone which was sufficient to produce systemic lesions in his rabbits?

Dr. Becker: We followed the dose reported by Rich, which is eight and one-third mg. (one-third cc.) of cortisone per day intramuscularly for 21 days.

Dr. Frederick Blodi (Iowa City, Iowa): The first question I would like to ask is whether Dr. Becker has had beneficial results from testosterone in human diabetic retinopathy.

Second, has Dr. Becker ever seen diabetic retinopathy develop in a patient with pernicious anemia who had cortisone?

DR. BECKER: I am sorry to say that, in answer to the first question, we have not been as successful as Saskin and his co-workers in alleviating diabetic retinopathy with the administration of testosterone. We have seen improvement in some cases, but not as often as they report. They reported 40 to 50 percent of patients improved. We failed to see that much improvement.

Patients with pernicious anemia who are treated with cortisone are extremely rare. I don't think I have ever seen one, nor have I ever seen any reports of their developing lesions.

DR. THOMAS TRAVERS (Australia): What was the reason for including aureomycin in the diet? How was it given?

DR. BECKER: Aureomycin is included in most rabbit feeds. It serves two purposes: It prevents infection in the rabbits, and, more significantly, it spares B_{12}, and perhaps other vitamins, too, for the rabbit.

Aureomycin apparently destroys some bacteria which feed on the B_{12} ingested, thereby permitting more B_{12} for the rabbit and less loss to the bacteria.

DR. I. S. TASSMAN (Philadelphia): I would like to ask if it is reasonable to assume that, in patients with pernicious anemia, the adrenocortical function might be normal and uninvolved, so that in those cases there would be an absence of a retinopathy?

DR. BECKER: That is a very good question, Dr. Tassman, and one that has puzzled us greatly.

Interestingly enough, patients with pernicious anemia not only do not have excessive adrenal function but, as has been reported by Strauss and as we have been able to verify in some cases, they have decreased adrenal function. They resemble the diabetic without retinopathy.

I did not go into that in this paper, but the diabetic without retinopathy demonstrates decreased adrenal function as compared to the nondiabetic. Pernicious anemia patients resemble diabetics without retinopathy in that their adrenal function is decreased; that is, as measured by the eosinophil response to ACTH, by lipoid vacuolization of the adrenal cortex, and by weight of the adrenals.

DR. PARKER HEATH (Boston): Is there any correlation between the microaneurysms that are seen in the optic-nerve lesions—vein occlusion, for instance—and adrenal changes or other chemical changes?

DR. BECKER: That is an excellent question, Dr. Heath. As a matter of fact, we have seen no correlation. Some years ago, with the help of Dr. Post, we were able to produce retinal microaneurysms in cats by means of experimental occlusion of the central retinal vein. Those cats, as far as we know, had nothing wrong with their adrenals. They were normal stray alley cats which, some two months after an eye operation involving occlusion of their central retinal veins, developed capillary aneurysms.

THE RECORDING OF THE ELECTRORETINOGRAM IN HUMANS AND IN ANIMALS*

Investigation of retinal sensitivity following brief flashes of light

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Introduction

Since Holmgren's[1] discovery of the retinal action potential in 1865, many investigators have studied the electrical response elicited upon stimulation of the eye by light. Studies have been made on human and animal eyes (vertebrate and invertebrate) to investigate problems of basic retinal physiology (for example, Granit,[2] Adrian,[3,4] Riggs and his colleagues,[5–8] and by Monnier[9]).

In recent years a great deal of clinical interest has been displayed in the human electroretinogram. This interest has kept pace with improvements in instrumentation, the use of the contact-lens electrode, and the increasing knowledge gained from basic retinal studies (for example, Karpe,[10] Bounds,[11] and Burian[12]).

Our interests lie in both clinical and physiologic aspects of the problem. Measurements designed to give information regarding the sensitivity of the retina under various conditions of light and dark adaptation may offer possibilities for a clear understanding of the peripheral nervous visual mechanism.

Our primary experimental interest at present is the study of the recovery of retinal sensitivity following a brief flash of light. The technique which makes use of a stimulus to test excitability following a constant conditioning stimulus has yielded significant information regarding the peripheral nerves and the central nervous system. Our preliminary results in the human eye show that the objective measurement of retinal sensitivity by means of electroretinography may be expected to furnish information regarding excitability of the retinal receptors unobtainable by other usual techniques.

Methods

The methods used in this study are similar basically to those reported by other investigators. The apparatus and techniques to be described have proven to be unusually flexible in the recording of the retinal electrical responses in animals and in untrained human subjects. The electroretinogram is recorded from the eye by means of two electrodes, one in contact with the cornea and one in contact with the skin near the eye. The action potential is led to a D.C. amplifier which leads in turn to a cathode ray oscilloscope upon the screen of which the recording is visualized. The eye is stimulated by light from a specially designed optical bench. Some of the details of the techniques and apparatus are described.

The corneal electrode

Our corneal electrode is similar to the one devised by Riggs.[5] It consists of a silver disc embedded in the temporal portion of the corneal section of a plastic contact lens. The lead from this is a fine flexible wire silver-soldered to the silver disc.

Our experience has shown that about 70 to 80 percent of the untrained human subjects can be fitted properly and comfortably by one of three lenses from a trial set. The three lenses have scleral curvatures whose radii are 12.25 mm., 12.5 mm., and 12.75 mm., respectively. Each lens has a corneal

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curvature of 7.5-mm. radius. The remaining 20 to 30 percent of subjects are fitted by trial lenses of other curvatures.

One or two drops of 0.5-percent Pontocaine (tetracaine hydrochloride) are instilled in both eyes of untrained subjects before the contact lens is inserted. Some of our trained subjects have individually fitted contact lenses and do not require a local anesthetic. We have not found lid akinesia or the use of a speculum necessary. Even with a speculum device, attempted squeezing of the lids caused artefacts in the tracing.

The indifferent electrode is a small silver disc held in place on the skin over the zygoma just below and temporal to the orbital margin.

The silver electrodes were chlorided to furnish silver-silver chloride electrodes.

The subject is grounded by means of a silver disc placed on the forehead or in the mouth between the buccal mucosa and the teeth. A silver-silver chloride wick electrode is used on the animal eye; the indifferent electrode is inserted in a small skin incision overlying the orbital rim.

The optical system

The optical system provides a flexible apparatus for applying accurately calibrated photic stimuli to the eyes of both animals and humans. Provisions are made to vary the intensity, the duration, the color of the light stimulus, the retinal location, and the size of the retinal area stimulated.

The optical system contains two light channels each of which can be varied independently as regards the above listed variables. The light from each channel is directed into the eye under study.

The stimulating apparatus is shown diagrammatically in Figure 1. Figures 2 and

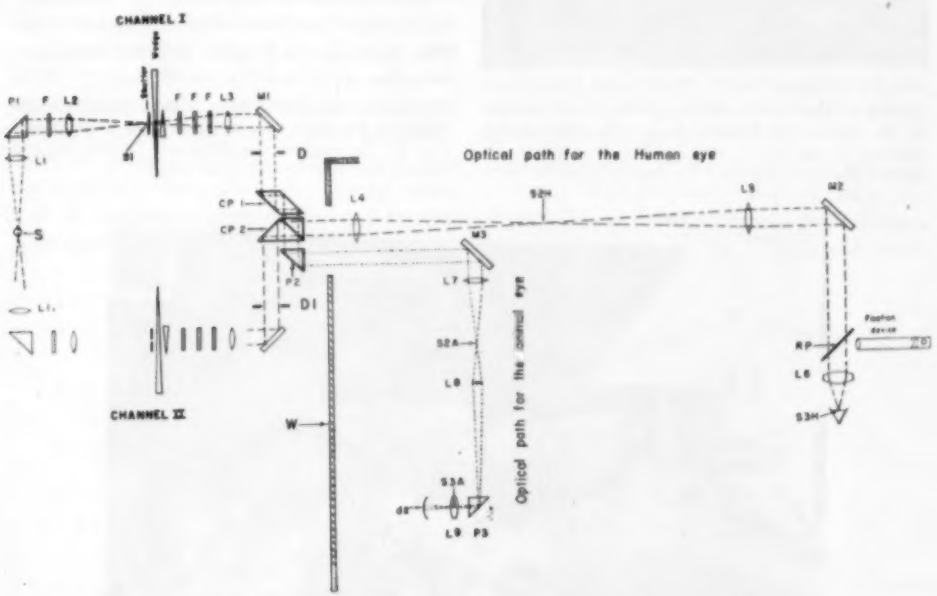

Fig. 1 (Wagman, et al.). A diagrammatic sketch (approximately to scale) of the optical apparatus used to stimulate the eyes of both humans and animals. A lens is designated by the letter L; a mirror by M; a prism by P; a filter holder by F. CP 1 and 2 are combining prisms. D and D1 are moveable diaphragms. S is the light source. All images of the S are designated by S followed by a number and letter. RP is a reflecting plate. d2 is an image of D and/or D1 at the eye of the animal. W is the wall of the shielded room. See text for detailed explanation.

Fig. 2 (Wagman, et al.). Photograph (taken from above) of that portion of the optical bench outside of the electrically shielded room (W indicates the position of one wall). Some of the components are labeled. Refer to Figure 1 for detailed identification.

3 are actual photographs with some components labeled to offer orientation in relation to Figure 1. The light source, S,* which is a coiled tungsten filament (18 amp., 6 volts) is set at the principal focus of lenses L1 and $L1_1$. In this way two symmetrical optical pathways (channels I and II, respectively) are obtained. The separate, symmetrical pathways are combined at the combining prisms (CP). From this point on they are identical.

Light from the source S in channel I is collimated by the lens L1, bent 90 degrees by the prism P1, and converged by lens L2. An image of S is thus formed at S1, at which point the shutter opening is placed. The light is collimated once again by the lens L3, bent 90 degrees by the front surfaced mirror M1, and directed into the combining prisms (CP 1 and 2). In channel II, the light path is similar in all respects up to this point.

The combining prisms (CP) recombine the light paths of both channels so that from this point on each path follows the same

* A small amount of light is led out through the front of the light source housing to a photo-cell (fig. 2). By reading the photo-cell current, we can monitor the light output at all times.

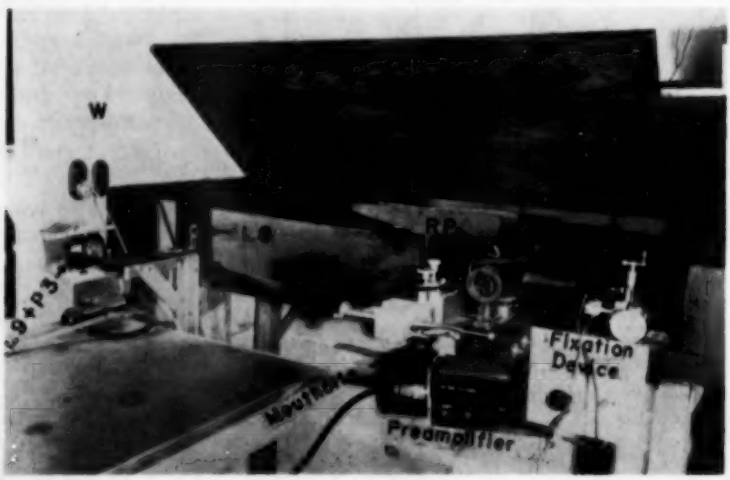

Fig. 3 (Wagman, et al.). Photograph of inside of electrically shielded room showing mouth-bite holder and its manipulator in addition to the appropriate parts which are detailed in Figure 1.

course. This system of reflecting prisms is a modification of the optical system used in the monobjective binocular microscope which is designed to provide identical images for both eyes when using a single objective. Here, of course, the light travels in a direction opposite to that in the binocular microscope head. The paths of the two parallel light beams from both channels are indicated in the diagram. The prisms are designed to provide identical overall distances for each light path.

The final reflecting surface of the combining prism CP 2 reflects half the light and transmits half the light from each channel. Therefore, reflected light from one channel is combined with the transmitted light from the other channel. Thus half of the total light is sent out along a line joining the source (S) with the midpoint of CP 2. This light is directed along the optical pathway for the human eye through an opening in the wall (W) of an electrically shielded room.

The remaining half of the light is sent from the combining system along a line at right angles to the combined paths for the human eye. The light is again reflected 90 degrees by prism P2 and is also directed into the shielded room along the optical pathway for the animal eye (see Figure 1 for the appropriate details).

In the optical system for the human eye, an image S3H of the source is finally produced in the pupil. The size of this image is 1.5 mm. by 0.8 mm., a smaller size than any pupil at a high brightness. Thus there is no necessity for an artificial pupil. The maximum visual angle is 30 degrees.

The eye sees an image of D and/or D1 at the point RP. The latter point is the first focal point of the ocular lens L6; therefore, rays entering the eye come from "infinity" and insure relaxed accommodation in the emmetropic eye. The field is seen as a Maxwellian view. The path of the light rays from D are shown in Figure 4-A.

At RP a thin plate of flat glass is inserted at an angle of 45 degrees to the optical axis to provide the subject with a fixation point. This point is projected onto the glass plate by means of the fixation device indicated in the diagram. The device is a small projector mounted rigidly on the tool post of a jeweler's lathe. The fixation point can be set accurately at any position in the visual field. The color and size of the fixation point can be varied by placing different filters and diaphragms in the projector.

In the optical system for the animal eye, an image of D and/or D1 is produced at the pupil.* This is shown schematically in Figure 4-B. A magnification of 1/10 is obtained;

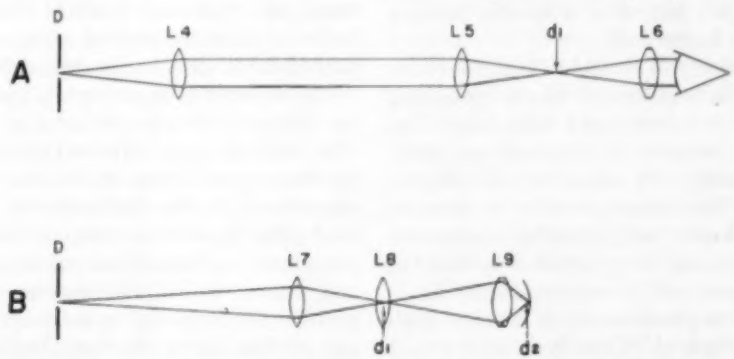

Fig. 4 (Wagman, et al.) A diagrammatic sketch (not to scale) of the path of the light rays from the diaphragm D (and/or D1) of Figure 1. (A) The light path to the human eye showing how an image of D is formed at the first focal point of the ocular lens, L6. The rays enter the eye from "infinity." (B) The light path to the animal eye showing the image (d2) of D formed at the eye of the animal. The numbers of the lenses correspond to those in Figure 1. See text for detailed explanation.

the maximum spot obtainable on the eye is five mm. in diameter. The prism P3 and the projection lens L9 are mounted together on a rotatory mount in such a way that the light can be projected around a complete circle with its radius perpendicular to the optical axis formed by the mirror M3 and lenses L7 and L8 (fig. 1).

At positions D and D1 there is provision for placing slides and diaphragms which can be made to suit many experimental plans. The slide is placed in a carrier which is moveable in a horizontal and a vertical direction, perpendicular to the optical axis. It may be fixed in any position in a reproducible manner. Thus it is possible to vary the area of retina stimulated over a wide range. (For the human the maximum visual field is 30 degrees as already mentioned.)

The part of the retina stimulated may be varied by moving the diaphragm and by changing the position of the fixation point. By using both channels I and II, two spots of light can be placed accurately on adjacent parts of the retina. Each spot is independently variable in regard to intensity, area, and color. For example, to perform a standard dark adaptation experiment, we may light adapt with a centrally fixated 30 degree field using one channel, and test the response with a small spot (for example, five-degree visual angle) located at a specific position within the larger field.

The intensity of the light (and, therefore, the apparent brightness as seen by the human eye) may be varied over a wide range. The minimum intensity is adequate to determine accurately the subjective dark-adapted threshold. The intensity is varied by means of a neutral density wedge (used in conjunction with a balancing wedge which is indicated in the diagram) and by neutral density filters which can be placed in any of the filter holders (F in Figure 1). Color is varied by means of either the Wratten monochromatic filter series (Numbers 70, 72A, 72B, 73, 74, 75, 76) or interference type filters.†

The duration of the flash may be varied within wide limits by means of an electronically operated shutter which is placed at S1, the position of the image of the source S in channel I. An identical shutter is placed at the corresponding position in channel II.

The versatility of the shutter (Husta and Wagman[13]) is an important feature of the apparatus. Its speed can be varied accurately from 1/1,000 second to 100 seconds in single exposures by means of electronically controlled selector switches on a remote control panel. A "bulb" selector is also present.

Another circuit provides variable delays between the two channels. Channels I and II can thus be operated individually, alternately, or simultaneously, with all the possible variations of exposure times noted previously.

Flash frequency up to 15 per second without distortion of the accuracy of exposure speed can be obtained. At a frequency of 20 per second there is slight loss of accuracy.

Photometric and relative energy calibrations are made by methods described previously (Hecht, Shlaer, and Verrijp,[14] Wagman and Gullberg,[15] Riggs, Berry and Wayner[7]). For white light the absolute value of brightness seen by the human eye was determined for one position of the wedge by means of a calibrated standard (11.3 millilamberts) set at the position of the reflecting plate (RP in Figure 1) (Hecht and Shlaer[16]).

The standard is so set that its light forms one half of a circular photometric field the other half of which is formed by light coming through the optical bench. The wedge is moved until the two half fields are equal in brightness. From the value of the wedge position and the density calibrations of wedge and neutral filters, the brightness at any position of the wedge in conjunction with any combination of filters can be calculated.

* When an open eye is used, the image is produced directly on the retina at the desired location.

† The interference type filters are always placed in the filter holder in the collimated beam between the prism P1 and the lens L2 of channel I and in the corresponding holder of channel II.

For accurate and reproducible observations in humans we have found the use of a rigidly fixed mouthbite to be an excellent aid. It can be made individually from dental compound in about two minutes. This is rigidly mounted on a micromanipulator constructed to move in three directions, thus insuring accurate and reproducible fixations and positioning of the light beam.

The recording system

The retinal action potentials which are picked up by the corneal and indifferent electrodes are led into a high gain direct coupled amplifier of high stability and a low noise level. The use of the direct coupled amplifier makes possible the recording of even very slow potential changes such as are seen upon prolonged light stimulation. From the amplifier the potentials are led into a cathode ray oscilloscope. The oscilloscope sweep is synchronized externally by a pulse from the electronic apparatus controlling the opening and closing of the shutters. In this way a single sweep is recorded on the stationary film.

The oscilloscope is of the double beam type. The second beam signals the opening and closing of each shutter. This is accomplished by leading into it pulses from the appropriate contacts of the shutter relays. Calibrations of the shutters by means of an accurate photocell showed that these electrical pulses give an accurate indication of the action of the shutters. In order to obtain accurate timing of events a time signal is put into the second beam at frequent intervals.

We are able to record the electroencephalogram simultaneously with the electroretinogram by using a separate amplifier (resistance capacity coupled amplifier with appropriate time constant).

The Grass kymograph camera which we use for making photographic records has provision for leading light onto the back of the recording film. We have two Hathaway mirror galvanometers set up to accomplish this. A light beam from each mirror makes a tracing on the back of the film while the light from the cathode-ray oscilloscope beam makes a tracing on the front of the film. In this way we can use four recording channels. When recordings are made in this way, the film strip moves continuously at a constant rate while the shutter remains open.

Experimental procedure and results

In a typical experiment the eye is dark adapted and then stimulated with an accurately controlled flash of light (the conditioning stimulus). After a delay of varying duration the eye again is exposed to another flash of given intensity (the test stimulus). This cycle is repeated every minute using a different delay interval between the conditioning and test stimulus. The interval is increased progressively until the test response reaches the height elicited by an unconditioned test stimulus.

With the brief flashes (0.01 to 0.05 seconds) and with moderate intensities the test response recovers well within five seconds following the first flash. We have found that one minute between cycles is more than adequate to insure the return of the sensitivity of the retina, as measured by the height of the conditioning response, to its basic dark adapted state.

Figures 5, 6, and 7 describe the results of a typical experiment on a human eye. Both conditioning and test flashes subtended a visual field of 20 degrees centrally fixated. In any one series the intensity of both stimuli remained constant throughout. Then the intensity of one or the other stimulus was changed and another series of measurements was made. Four series were run in the experiment described.

In Figure 5 are shown a few records from a typical series. The experiment and some of the results are outlined in the legend for the figure. It is seen that when both conditioning and test stimuli are kept constant in brightness (each is of different brightness in this study) the height of the test response de-

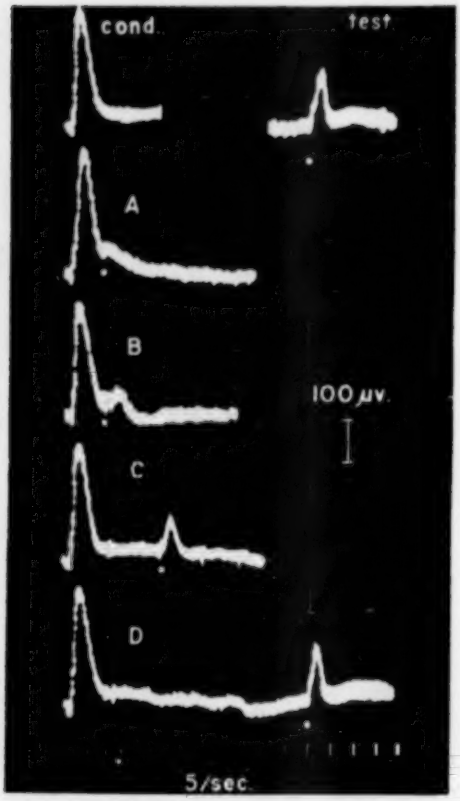

Fig. 5 (Wagman, et al.). A series of human electroretinograms obtained from the dark-adapted eye showing the progressive increase of the amplitude of a test response when it follows a conditioning response at increasingly greater time delays. The flash duration for both conditioning and test stimuli was 0.02 second. The brightness of the former flash was 132 millilamberts and for the latter was 18 millilamberts. The upper records show conditioning and test responses elicited separately. The respective heights in microvolts are 309 and 123. The conditioning flash is coincident with the beginning of the sweep while the moment the test flash was given is marked by means of a dot in the appropriate place.

The measurements from the records are as follows:

Record	Interval between Flashes (seconds)	Height of Test Response (microvolts)
A	0.18	8
B	0.23	39
C	0.63	80
D	1.75	120

pends upon the interval between the conditioning and the test stimuli.

Figures 6 and 7 summarize the results obtained from one subject during one experimental period. (The records of Figure 5 give some of the data which are plotted as curve B of Figure 6.) Figure 6 shows the recovery of the test response amplitude following three different conditioning stimuli brightnesses. The necessary information is summarized in the legend of the figure.

Several facts became evident in the course of this experiment. The brighter the initial stimulus, the longer it took for the reappearance of the electroretinogram elicited by the test flash. Thus when the initial stimulus brightness was 795 millilamberts, the second flash produced no electroretinogram 0.35 seconds later (Curve C); whereas, when the initial stimulus brightness was lowered to 132 millilamberts, a second flash at 0.18

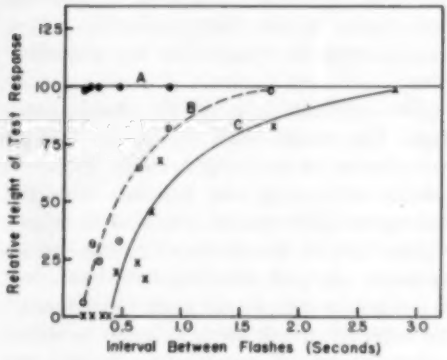

Fig. 6 (Wagman, et al.). The relationship between the relative height of the test response obtained from a human dark-adapted eye and the interval between the conditioning and test flashes of light. The three curves, A, B, and C, show the recovery of the amplitude of the test response following conditioning stimuli of brightnesses 10, 132, and 795 millilamberts respectively. The brightness of the test flash (18 millilamberts) was kept constant throughout. The amplitude of the response elicited by this flash in isolation is given a value of 100 (= 122 microvolts). The response to the first conditioning flash was 122 μv for Curve A, 309 μv for Curve B, and 347 μv for Curve C. (Curve B is plotted in part from the records of Figure 3.)

seconds already produced a measurable electroretinogram (Curve B).

Secondly it is seen from Figure 6 that the brighter the conditioning stimulus, the slower is the recovery of the test response to its unconditioned height. Figure 7 further illustrates this effect. Here the Curve A is the same as A in Figure 6. Now with the brightness of the conditioning stimulus unaltered, the test stimulus was reduced in brightness and curve D obtained. The striking difference in recovery of sensitivity is seen clearly.

Lastly it was noted that with the shortest interval between flashes (0.1 second) and the brightnesses of the stimuli employed here the subject reported that he saw two flashes even when the second flash did not elicit an electroretinogram.

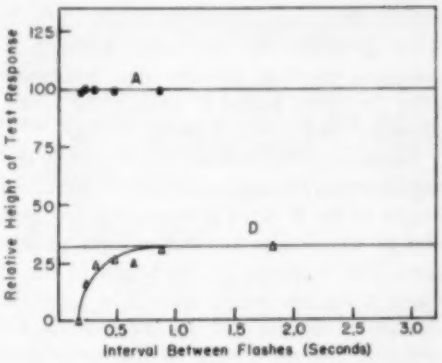

Fig. 7 (Wagman, et al.). Curves plotted in the same manner as those of Figure 4. Here A is the same curve as A of Figure 4. Curve D shows the recovery of the test response to its unconditioned height (40 μv or 33 percent of test height of Curve A) after lowering the brightness of the test flash to 1.8 millilamberts. The brightness of the conditioning stimulus was the same for both series of experiments.

Discussion

The results reported here, although of a preliminary nature, can be explained on the basis of our present knowledge of retinal physiology and of the photochemistry of vision. Assuming that the retinal action potential represents a measure of retinal sensitivity, we can see that even a brief flash of light may depress retinal sensitivity for a period of time.

The extent and duration of the lowered retinal sensitivity depend upon the intensity of a flash of constant duration, that is, the more intense the flash, the slower is the recovery of the sensitivity as measured by height of the retinal action potential. These short-duration effects resemble the long-duration changes in dark adaptation following various degrees of light adaptation.

In our experiments the flash, even the brightest one employed, produced comparatively small degrees of light adaptation, and consequently comparatively quick recoveries.

It seems that the height of the B wave of the electroretinogram represents a measure of retinal sensitivity, probably an index of the scotopic function of the eye.* We find, however, a discrepancy between the subjective and the electrical responses. The subject always noted two flashes of light even when the second did not elicit an electrical response.

Karpe and Tansley,[17] and Johnson[18] in their respective reports on dark adaptation also point out similar instances of nonparallelism. This may be due partly to the limitations of the recording apparatus.

Keeping this in mind, any quantitative interpretation of the electroretinogram must be carefully appraised. Undoubtedly further experimentation will evaluate its advantages and disadvantages in studying human retinal physiology.

Our results throw additional light upon the problem of fusion frequency as determined by electroretinography.

Cooper, Creed, and Granit[19] reported that the fusion frequency of the electroretinogram in the human always is less than that frequency at which the subjective sensation of flicker disappears.

Burian[12] showed that with increasing fre-

* In this preliminary investigation we have made no attempt to isolate the small photopic component of the electroretinogram from the main scotopic component.

quency above one/second, the height of the B wave progressively decreased, although it remained constant for any one frequency after the initial higher B wave of the series. Monnier[9] had reported similar results.

Burian's interpretation is borne out by our experiments. He suggested that the decreased height of the B wave at increasing frequencies of stimulation was due to light adaptation. The constancy of the height of the B wave in any one series, elicited by a specific frequency, means that the flash finds the retina in the same state of sensitivity each time.

The results of Burian are not in agreement with those of Euzière, Passouant, and Cazaban[20] who reported that up to four flashes per second, the height of the B wave remained normal. When they (Euzière, et al.) used four to six flashes per second the B wave disappeared. These latter authors also showed by using double flashes of the same intensities separated by varying intervals that the second electroretinogram could be elicited in normal form only if separated from the first by at least 300 milliseconds. The discrepancy between the results of Burian and those of Euzière and others possibly could be due to the fact that the latter authors used a more intense stimulus.* A very strong stimulus causes a marked decrease in retinal excitability, but on the other hand it offers a strong test stimulus to the retina.

The results of Dodt[21] are comparable. He showed that the fusion frequency for the light-adapted eye, determined subjectively and by means of the electroretinogram, was the same (upper limit of 60/second) provided that strong enough stimuli were used for recording the electroretinogram.

Discrepancies in the relationship between the subjective and electroretinogram fusion frequencies reported in the literature may be explained partly by different interpretations of fusion as determined from the electroretinogram and partly by the use of different light stimuli (for example, intensity, duration, dark-light ratio, color, and area subtended). Our results are not sufficiently detailed to warrant using them for any further analysis.† It should be emphasized, however, that they do point to the importance of the intensity of the stimulus in evaluating problems of fusion frequency.

Finally, we wish to comment on an idea which Burian[12] has noted, namely that the full value of clinical electroretinography cannot be realized until more standard functional electroretinographic tests have been developed. Our experimental plan, as reported in this paper, is designed to furnish information regarding a dynamic function of the retina. Although we cannot define it in specific terms, it is possible that measures of sensitivity, as made here, may in the future yield important information.

Perhaps the B wave elicited from an eye at the end of half an hour in darkness may be normal as we understand it now. However, if the height of the B wave can be used as a measure of retinal sensitivity, its depression and rate of recovery to normal following a brief flash of light may depend upon factors other than those responsible for its maximum height in the dark-adapted eye. We have been studying the electrical responses in normal individuals of different age groups by the method described above in order to establish normal values. Once a norm is established we intend to investigate clinical conditions to determine whether excitability changes different from those found in the normal eye exist.

Summary

1. A method of recording the electroretinogram in humans and animals is de-

* Their intensity, 500 lux at the eye, is a very intense stimulus. Burian does not give values for his intensities.

† It should be pointed out that in determining fusion frequency all stimuli in the series are of the same intensity. Even though our experiments were made by means of two stimuli of different intensities, the interpretations given here are quite valid for the reasons discussed.

scribed. The optical bench and accessories provide a versatile and flexible means of utilizing accurately controlled and positioned light stimuli.

2. By utilizing the method of conditioning stimulus followed by a test stimulus, an objective method of measuring retinal sensitivity is obtained as determined by the height of the B wave. It was found that the rate of recovery of the B wave height following brief flashes of light is dependent on the intensity of both conditioning and test stimuli.

3. In the very early period of recovery, a test flash could be seen by the subject without producing a measurable electrical response.

4. The significance of these results is discussed especially in relation to the determination of flicker fusion by means of electroretinography.

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ELECTRORETINOGRAPHY IN CASES OF NIGHT BLINDNESS*

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INTRODUCTION

The various forms of night blindness are usually characterized by (1) unusually high final thresholds when tested under low levels of illumination, and (2) a reduction in the ability to dark adapt as indicated by adaptometer tests. These findings are sometimes supported by clinical or postmortem examination of the retina. The present paper deals with still another method of studying night blindness, namely that of measuring the electrical response of the retina to stimulation by light. This response, the electroretinogram or ERG, is now relatively easy to measure in human subjects by the use of modern amplifying and recording equipment.

The electroretinogram, described by Holmgren[1] in 1865, has since been studied extensively in many animals including man. It represents a mass action of large numbers of retinal elements and is conveniently recorded between electrodes placed anterior and posterior to the retina. The response may be obtained from the freshly excised retina alone, from the excised eyeball, or from the intact eye. Degenerative changes are typically rapid in the excised preparations. The most satisfactory results are obtained therefore in intact eyes with a critical electrode firmly attached to the cornea and a neutral electrode located elsewhere on the head.

In lower animals, satisfactory results have usually been obtained by immobilizing the animal through the use of drugs or decerebration, and applying a wick electrode to the cornea.[2-4] For human beings a convenient technique described by Riggs,[5] in 1941, is to support the corneal electrode on a conventional plastic contact lens. The neutral electrode is worn as a headband by the subject. In this way it is possible to obtain electroretinographic records over a period of several hours without discomfort to the subject, thus making possible the study of slow effects such as those of dark adaptation.

Recent experiments have shown that the major part of the human electroretinogram, known in the literature as the "*b*-wave," originates in the peripheral, scotopic mechanisms of the retina. Thus a spectral sensitivity curve determined by measuring the height of the *b*-wave is similar in form to the conventional scotopic curve.[6]

Dark adaptation, too, affects the *b*-wave in a manner suggestive of the "rod" portion of the typical adaptometer curve.[7] And scattered light, falling upon peripheral receptors, certainly accounts for much of the electroretinogram as it is usually produced.[8, 9]

It is evident, then, that the electroretinogram is an appropriate indicator of processes such as dark adaptation involving principally the scotopic mechanisms of the eye. Only with special procedures is it possible to obtain photopic responses in this way,[10] and no attempt has yet been successful in recording from the fovea alone.[11, 12]

The electroretinogram is not presently very useful in relation to measurements of acuity, refractive condition, or the study of isolated visual fields. Clinically, the electroretinogram appears to be of greatest value in revealing the impairment of peripheral mechanisms involving the retina as a whole.

A number of investigators have already used the electroretinogram as a clinical test for night blindness. Karpe, for example, has reported that the electroretinogram is "extinguished" in cases of retinitis pigmentosa,[13, 14] siderosis,[15] and congenital night blindness.[13] Schubert and Bornschein[16] report a "total

* From the Psychological Laboratory of Brown University. This research was carried out under contract between Brown University and the Office of Naval Research, Department of the Navy.

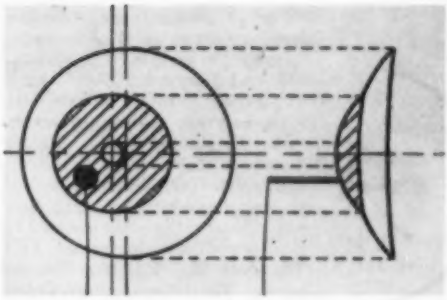

Fig. 1 (Riggs). Front and side views of a contact lens adapted for use as a mounting for a corneal electrode (from Riggs, 1941).

lack of the *b*-wave" in a case of congenital night blindness. Their published records show, for this subject, a large initial negative component followed by a brief positive deflection.

The purpose of the present paper is to add some cases to the literature from among our experimental subjects. Our interest has centered about the responses of normal eyes, but we have used cases of peripheral or macular degeneration to clarify our thinking about the scotopic and photopic portions of the normal response.

Method

A plastic contact lens is fitted to one eye of the subject.* The lens is then prepared to serve as an electrode in the following way[5] (fig. 1). A three-mm. hole is drilled through the lens at a point near the nasal margin of the corneal bulge (shaded area in the figure). A silver disk is then machined to a tight press fit in the hole. Before insertion, however, a short length of stiff brass wire is soldered to the silver disk at one end and to a 10-cm. length of fine enamelled copper wire at the other end. The disk is then pressed into the hole far enough so that it is slightly imbedded with respect to the inner surface of the lens.

A thin coating of household cement is then applied to seal the outer surface of the silver to the plastic lens and to serve as electrical insulation over the length of the stiff brass wire. After the cement dries, a coating of melted beeswax is added to provide further insulation and support, and to assure a smooth surface over the soldered connections.

The inner electrode surface is then chlorided by using the inverted contact lens as a cup filled with dilute NaCl solution. For this purpose a current of about 1.0 ma. is allowed to flow between a silver wire cathode dipped into the solution and the silver electrode for a period of about five minutes. The electrode is then ready for use. A standard contact lens solution is used when the lens is inserted.

Figure 2 shows the electrodes in position for recording. A loosely fitting headband is used to support the neutral electrode, a small rubber cup filled with a piece of saline-soaked cloth, contact with which is made through a tightly fitting silver rod, chlorided in the same way as the one in the contact lens. The headband is also used to support binding posts through which the electrode leads are connected to an amplifier input cable. With a well-fitted contact lens the subject is usually able to observe for two to four hours without discomfort. The eye is free to move, and the lids close easily

Fig. 2 (Riggs). Electrodes in position for recording the electroretinogram.

* Contact lenses were fitted by Edward J. Troendle, O.D., of Providence.

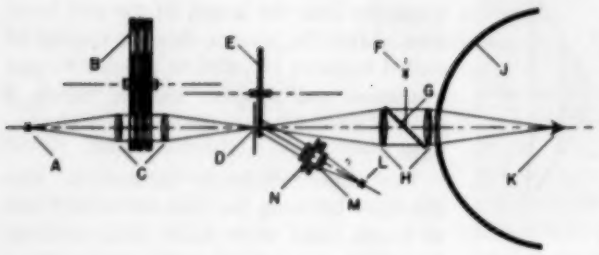

Fig. 3 (Riggs). A schematic drawing (not to scale) of the apparatus used to provide visual stimulation. (A) Tungsten filament source. (B) Filters in rotary mounts. (C) First pair of collimating lenses. (D) Diaphragm. (E) Rotary shutter or flicker vane. (F) Fixation point. (G) Glass plane for viewing fixation point. (H) Second pair of collimating lenses. (J) Bowl-shaped adapting screen. (K) The eye. (L) Tungsten filament source for illuminating flicker vane. (M) Collimating lens. (N) Variable density wedges (from Riggs, Berry, and Wayner, 1949).

over the wax-coated lead from the contact lens.

A direct-coupled amplifier[17] with a differential input is used to measure the potential difference between the eye and head electrodes. The output of the amplifier is used to drive a recording oscillograph of the loop magnetic type. Continuous photographic records are made of all responses, so that measurements can later be made of the amplitude, latency, and wave form.

A suitable optical system (fig. 3) provides an adapting field of light, together with test flashes of controlled area, intensity, duration, and wavelength. A more complete account of this system may be found in an earlier paper.[6]

In a typical experiment on dark adaptation, the electrodes are applied to the subject and he is allowed to enter a light-tight, electrically shielded room. He is given a half-hour of dark adaptation, followed by 15 minutes' adaptation to a large, uniform field of light. The adapting light is then turned off, and the subject gets in position for recording, holding as steady as possible and fixating on a small red fixation point. Test flashes are then administered at intervals during the course of dark adaptation, and the resulting electroretinogram responses are registered photographically.

Results

Normal dark adaptation

A normal electroretinogram from a dark-adapted eye is shown in Figure 4. This is a response to a 0.04 second flash of white light. The patch of light, centrally fixated, covers a circular area 12 degrees in diameter at an intensity of approximately 20,000 foot-lamberts. The initial effect of the light is seen to be a small negative dip known as the *a*-wave. This is followed immediately by the large *b*-wave amounting to nearly a millivolt under these conditions. After the decline of

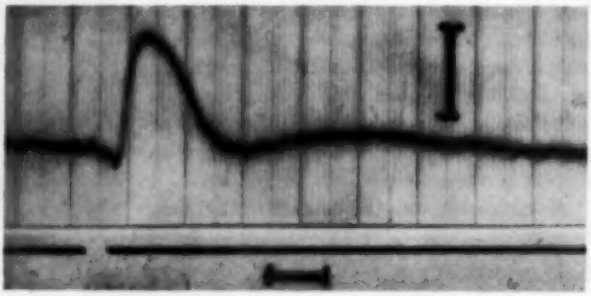

Fig. 4 (Riggs). A normal electroretinogram from a dark-adapted eye. The short vertical line represents 500 microvolts. The short horizontal line represents 0.1 sec. The signal denoting the 0.04 second flash is the short gap in the horizontal line. White light, intensity approximately 2×10^4 foot-lamberts.

the *b*-wave there is a second rise, the *c*-wave, which is typically small and of long duration.

The height of the *b*-wave has been shown[7,12] to provide a suitable measure of scotopic activity. During dark adaptation this height increases markedly in the normal eye as shown by the upper curves in Figure 5. This means that after an hour or two of dark adaptation a flash of 200 foot-lamberts is capable of arousing as large a *b*-wave as did a 20,000 foot-lambert flash at the beginning of dark adaptation.

SUBJECT G (CONGENITAL NIGHT BLIND)

This subject is congenitally night blind.* Electrical (*b*-wave) measurements of his dark adaptation are shown in the lower curves of Figure 5. It is clear that this subject shows but a small increase in electrical response as a result of dark adaptation. Furthermore, the intensity of light necessary to arouse a moderate (200 μv) response in this subject is about 3.2 log units higher than for a normal dark-adapted subject. Even in the early stages of adaptation, this subject is about 1.5 log units less sensitive than normal. For him, the total increase in sensitivity during dark adaptation is about 0.3 log unit as compared to a normal gain of about 2.0 log units.

Sample electroretinograms of Subject G are shown in Figure 6. These are some of the responses on which the curves of Figure 4B are based. As compared with normal responses of the dark-adapted eye (see fig. 4 and 8C) those of Subject G are smaller, require a higher intensity of light to produce them, and reach an earlier peak. In all these respects they appear more like the responses of a light-adapted eye than those of a dark-adapted one.

Adaptometer data on Subject G are shown in Figure 7. This graph is based on psychophysical threshold measurements taken by Dr. Sloan in the Wilmer Institute. It shows that even the "cone" or "photopic" portion of the curve is about 1.3 log units above that of the standard observer.

The "rod" or "scotopic" portion shows little adaptation, so that the final level is about 3.5 log units higher than the normal threshold intensity.

It may be noted that these figures agree rather well with the corresponding ones obtained in our laboratory by electrical measurements.† In both cases the conclusion is

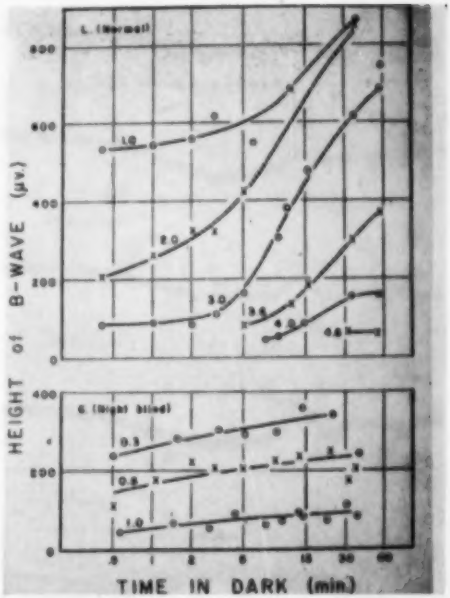

Fig. 5 (Riggs). Height of the *b*-wave of the electroretinogram as a function of time in the dark. Curves for a normal subject appear above, and those for Subject G (night blind) appear below.

* This subject was sent to us through the kindness of Dr. Louise L. Sloan of the Wilmer Institute of Ophthalmology of The Johns Hopkins Hospital and University, Baltimore, Maryland. We are indebted to Dr. Sloan for adaptometer data on this subject.

† The stimulating conditions were different in the adaptometer tests at the Wilmer Institute and the electrical recording at Brown University. It is believed, however, that in both cases the responses originate from receptors in the peripheral retina. The adaptometer data are for a one-degree white test patch at 15 degrees from fixation in the nasal field after three minutes' light adaptation to 1,100 ml. The electrical data are for a 7.5 degrees white test field centrally fixated after 10 minutes light adaptation to 170 ml. Because of the scattered light

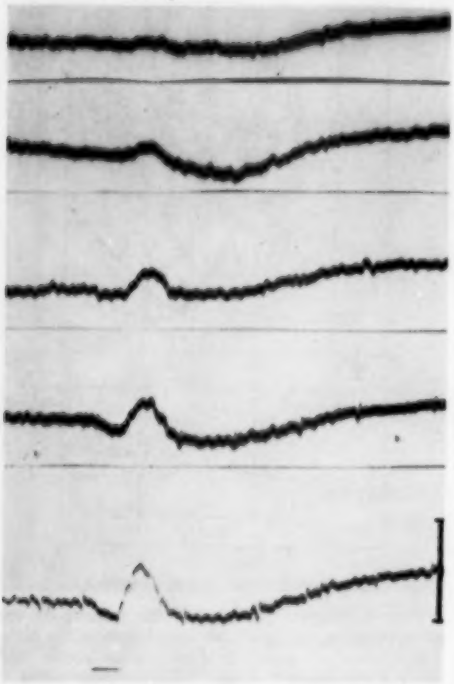

Fig. 6 (Riggs). Responses of Subject G (night blind) to increasing intensities of white light. Highest intensity is for the bottom record, approximately 2×10^5 footlamberts. The flash of 0.04 second is signalled by the short horizontal line. The notched line indicates time in 0.1 second units. The short vertical line represents 500 microvolts.

that Subject G shows very little dark adaptation, and that his vision appears to resemble that of a light-adapted eye with little, if any, use of scotopic mechanisms.

effects noted above, the electrical responses are largely those from peripheral regions.

* Subjects F and L were made available through the courtesy of Dr. F. D. Carroll, College of Physicians and Surgeons, Columbia University, New York, New York. Dark-adaptation measurements made on these observers by Dr. C. Haig, Department of Physiology and Pharmacology, New York Medical College, New York, New York, gave evidence of only cone activity. The Purkinje phenomenon was absent. Clinical studies also indicated a complete lack of rod function. The assistance of Dr. John C. Armington in recording the electrical responses from these subjects is gratefully acknowledged.

Subjects F and L (Congenital night blind)

These subjects, sisters, are of a family in which night blindness is hereditary.* In this respect they differ from Subject G, in whose case no near relatives seem to be affected. They also differ from Subject G in the fact that their photopic thresholds are not much higher than normal. Subject F has essentially normal photopic vision, which Subject L has thresholds about 0.5 log unit above normal.[18] Like Subject G, however, they appear to be almost completely lacking in scotopic vision. Adaptometer tests by Dr. Haig in New York showed an absence of the rod portion of the dark-adaptation curve.

Figure 8 shows electrical responses from night-blind subjects F and L, together with those of a normal subject, C. Again it is clear that the night-blind subjects have shown relatively little gain as a result of dark adaptation.

During light adaptation to a large field at 46 ml. the responses of Subjects F and L are no smaller than those of C, the normal subject. After five minutes of dark adaptation, however, the normal response has attained a height greater than any of which the night-blind subjects are capable. With further time in the dark the normal subject continues to show a marked increase

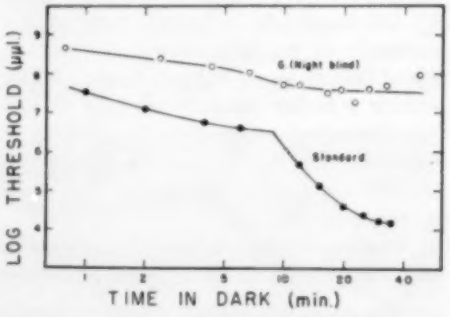

Fig. 7 (Riggs). Adaptometer data obtained by Dr. Louise Sloan on Subject G (night blind) and on standard observers.

in height of response. The night-blind subjects, on the other hand, show little development of the *b*-wave beyond the first few minutes. In this respect the electrical data are in good quantitative agreement with the adaptometer data obtained by Dr. Haig.

SUBJECTS S, B, AND R (RETINITIS PIGMENTOSA)

These subjects were identified clinically* as cases of retinitis pigmentosa.

Subject S represents an early stage of degeneration. The pigment spots in his eye are very small. He appears to have relatively good vision, particularly in the central part of the eye. He is able to dark adapt down to a level which is within normal limits of the dark-adapted threshold.

Electrical responses were elicited from Subject S by the usual procedure of applying short flashes to the dark-adapted eye. It was found that our highest stimulus intensity (approximately 2×10^6 foot-lamberts in a 12-degree test field, centrally fixated) was required to elicit even minimal responses from the retina. This intensity is approximately 4.5 log units higher than normal for these very small electrical responses. No responses whatever are elicited until after 20 minutes or more of dark adaptation. The only feature of the electrical response which appears to be normal is that of a relatively slow "*c*-wave," which we have often found to be correlated with the pupillary mechanism rather than the retina itself.

With respect to Subject S, we must reach the surprising conclusion that an eye which is functioning almost normally can suffer a nearly complete loss of the electrical response of the retina. This suggests that the mechanism responsible for the electrical retinal response in the normal eye is damaged very early by retinitis pigmentosa.

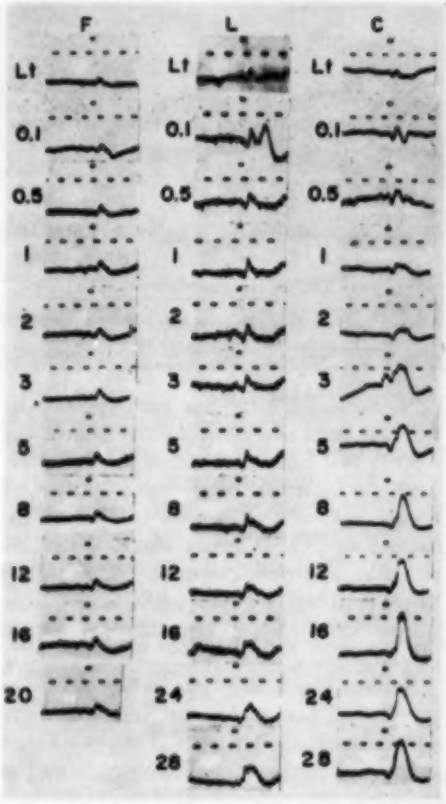

Fig. 8 (Riggs). Responses of Subjects F and L (night blind) and Subject C (normal) during dark adaptation. Uppermost record in each case shows the response during light adaptation. Lower records are for times (minutes) in the dark indicated by the figures to the left of each record. Dashed line indicates tenths of seconds. Short line at the top of each record indicates moment of the 0.02 second flash.

Subject B represents a more advanced case of retinitis than Subject S. Figure 9 shows, in the upper record, a response from the eye of Subject B to our highest intensity of white light. The lower record is the response of a normal subject, M, to a flash of white

* Subjects S, B, and R are patients of Dr. Dan M. Gordon of New York. They came to our laboratory for electrical tests at the suggestion of Dr. Roy G. Hoskins of the Boston Branch, Office of Naval Research. Clinical findings on these patients were made available to us through the kindness of Dr. Gordon. Electrical responses of these subjects were recorded with the kind assistance of Dr. Robert M. Boynton.

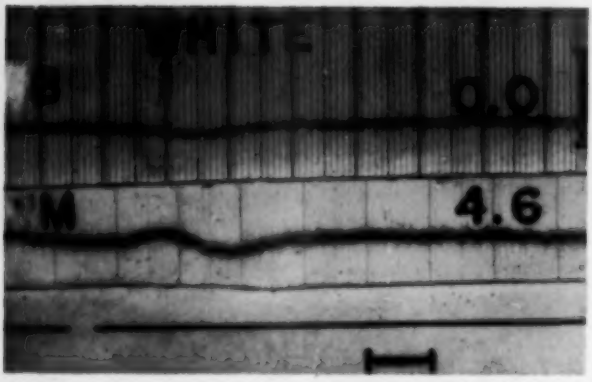

Fig. 9 (Riggs). Response of Subject B (retinitis pigmentosa) to white light of high intensity and the response of Subject M (normal) to a much weaker flash. The short vertical line represents 500 microvolts. The short horizontal line represents 0.1 seconds. The signal denoting the 0.04 second flash is the short gap in the horizontal line.

light whose intensity has been reduced by 4.6 log units. It is apparent from these records that Subject B shows almost no response in the nature of a *b*-wave, but that his *c*-wave response is similar to that of the normal subject. We may conclude that, in terms of electrical response, Subject B is more than 4.6 log units less sensitive to stimulation by white light than the normal subject.

Subject R is a still more advanced case of retinitis pigmentosa. Foveal vision is beginning to be impaired. Electrical responses were almost completely absent. Subject R is like the other two retinitis cases in exhibiting a *c*-wave of considerable size. There is a latency to peak of 0.35 second approximately.

In the case of Subject R we also did an experiment in which electrodes were placed in a position often used to measure eye movements. The electrodes were located on the temporal bones in positions near each of the eyes. Under these conditions, Subject R showed a potential shift of about 40 microvolts accompanying a 15-degree eye movement laterally.

Three normal subjects under corresponding conditions showed shifts of 117, 72, and 107 microvolts respectively. It was concluded that very likely the "resting potential" of the eye of Subject R had suffered a diminution accounting for the small size of these responses. It was also observed that normal eye movement records associated with reading were clear and easily measurable in the control subjects but were totally absent in Subject R.

Cases of macular degeneration

In the cases of night blindness there is impairment of the rod or scotopic mechanisms. It is of interest to contrast these with cases in which night vision is normal but day vision is seriously impaired. Two such cases have come to our attention. In each case there is macular degeneration of such degree that acuity is very poor and reading is difficult or impossible.

With each of these cases the electroretinogram is found to be nearly normal. This is true even when the defective macula alone is directly stimulated by the test light. The result is similar to the finding, with normal subjects,[9] that stimulation of the blindspot produces so much scattered light that electrical responses of normal amplitude are found.

Discussion

Something of a problem is presented by the electrical responses of the congenitally night-blind subjects. It is true that these responses are smaller than those of normal persons, and they require much more intense stimuli for their arousal. Yet the form of the response is rather similar to that of the normal, particularly in the light-adapted state.

If these subjects are indeed lacking in rod

function, as the clinical findings would indicate, this means that such electrical responses as we do obtain are those originating with the cone receptor system. Against this view, however, is the fact that a consistent, though small, increase in response occurs during the later stages of dark adaptation. It cannot now be decided whether some slight scotopic activity is present in these patients or whether photopic adaptation continues beyond the usual five or 10 minutes in the dark.

Still more puzzling is the lack of electrical responses in cases of retinitis pigmentosa. Since macular vision is unimpaired and some peripheral vision also exists in two of these cases, it is clear that the disease has caused a specific blocking of the electroretinogram. A possible explanation of this phenomenon lies in the fact that the disease manifests itself by widespread perforations of the retina. Such perforations may conceivably result in a short-circuiting of the potentials arising in the retina so that they are no longer recorded from the remote leads on the eye.

An attempt was made to test the hypothesis of electrical short-circuiting in experiments on the frog retina.[10*] Electrical responses were recorded before, during, and after a series of five perforations were made in the retina. The effect of the perforations was to decrease the magnitude and increase the latency of the *b*-wave. The *a*-wave was less easily affected, but eventually suffered a similar decline if the perforations were continued for a long enough time. These experiments suggest that the *b*-wave may be dependent on intactness of the retina when it is recorded by means of remote electrodes. They do not provide, however, any further explanation of the loss of electroretinogram in cases of retinitis pigmentosa.

In summary, then, we have found substantial agreement between adaptometer data and electrical response data, with cases of congenital night blindness. In retinitis pigmentosa, however, the electroretinogram is practically absent even in cases of relatively slight impairment of vision. The electroretinogram is evidently useful as a sensitive test for retinitis pigmentosa, as Karpe[13, 14] has pointed out. In certain other cases the electroretinogram data may perhaps be useful in distinguishing between retinal impairment and disorders of the higher visual centers.†

Brown University (12).

* These experiments were conducted by Mr. William Bush.

† Recent experiments[20] have shown, for example, that protanopia (hereditary red-blindness) is of retinal origin. This condition is accompanied by a characteristic loss of an early component in the electroretinogram when red light is used as a test stimulus.

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THE ORIGIN OF THE ELECTRORETINOGRAM*

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Everywhere in the nervous system electrical reactions serve as the most refined means for the study of the organization and mechanisms of nervous functioning. Applied to the retina, electrophysiologic methods have been most successful in providing concrete evidence, by measuring single cell reactions, of the pattern of nervous activity produced by retinal stimulation of different intensity, duration, and hue.

Those electrical phenomena of the retina, which are longest known and most easily measurable, are, however, still obscure as to the site and mechanism of their generation. This holds true for the steady potential difference across the vertebrate eye and for the electroretinogram which is defined as the retinal response to illumination measured from cornea (or corpus vitreous) in reference to the outside of the eye.

Both phenomena must be considered the best sources of objective informations concerning the general state of retinal function under normal or abnormal conditions. Moreover, due to their pick-up from points distant to the photoreceptors and nerve cells, these phenomena offer a number of electrophysiologic problems which if solved may well lead to a better understanding of similar electrical manifestations measured from the brain and other sense organs.

The following is a brief account of experiments designed to analyze the various components of the electrical potentials across the eye as to localization and mechanism of generation.† The analysis is based upon the action of intravenously administered agents as ascertained by the structural changes they produce. The first of the agents studied extensively is iodoacetic acid which in its actions upon cellular metabolism singles out the visual cells as those affected in the most striking and damaging manner (Noell, 1951-1953).

In rabbit and cat, the poison abolishes vision and electrical reactions of the optic pathways in response to illumination within a few minutes after the injection. Moreover, if the spontaneous elimination of the poison is delayed by a second injection and its effect on the visual cells thus prolonged, irreversible

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† A complete account of these experiments is contained in "Studies on the Electrophysiology and the Metabolism of the Retina," October, 1953, p. 122, Project Report, USAF School of Aviation Medicine, Randolph Field, Texas.

cell damage ensues and visual cell death occurs within a few hours.

Histologic examination of such eyes several weeks after the injections reveals widespread or complete disappearance of the visual cells while the inner layers are generally well preserved. The pigment epithelium is not affected when doses of iodoacetic acid are employed which just suffice to destroy the visual cells; however, when higher doses are used, the widespread disappearance of the visual cells is associated with localized degeneration of the pigment epithelium.

An equally useful tool as iodoacetic acid for the analysis of retinal potentials was found in sodium iodate (Noell, 1953). A single injection in albino rabbits produces widespread deterioration of the pigment epithelium associated with the degeneration of the sensory organelles. The latter effect involves first the outer and later the inner limbs. Two to three weeks after the injection of four to six cc. of a two-percent sodium iodate solution, the retinal layers vitread the external limiting membrane are preserved extensively while practically the whole pigment epithelium is destroyed; the outer limbs have then disappeared but remnants of the inner limbs are still present.

Azide response

The starting point of my analysis is a peculiar reaction of the steady potential across the eye in response to the injection of sodium azide (Noell, 1952c). In the experiment of Figure 1, the potential between cornea and posterior pole of the atropinized eye is recorded by direct-current amplifier and cathode-ray in a curarized and artificially ventilated rabbit. In response to the rapid, intravenous injection (hind leg) of one mg. of azide (NaN_3), the potential difference across the eye suddenly increases for a short period of time to values considerably exceeding those produced by illumination and more than doubling the normal size of the potential across the eye.

	b wave mV	1 mg. NaN_3 mV	outer nuclear layer %	pigment epithelium %	bipolar cells %
control	0.25	10.0 (7.5–11.5)	100	100	100
3-9 weeks after IAA n=17	0	3.8 (0.2–6.5)	2	81	80
2-3 weeks after Iodate n=19	0.04	0.03 (0–0.2)	85	< 20	98

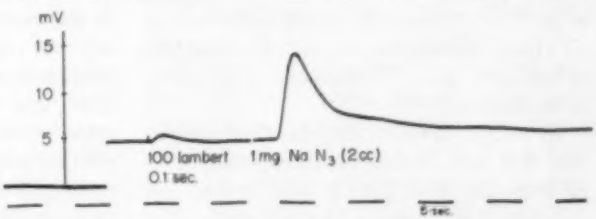

Fig. 1 (Noell). *The direct-current potential rise in response to sodium azide.*

The record (lower half of figure) starts with the amplifier input shunted; at the first break of the curve the electrodes are connected with the recording system. They measure the steady potential difference across the eye. A single, strong flash of light of 100 lamberts of 0.1-second duration produces the subsequently recorded variation of the potential difference. At the following short break of the curve, 1.0 mg. sodium azide dissolved in 2.0 cc. salt solution is injected into a hind leg vein. After a short delay (circulation time) the potential suddenly rises for a short period of time by about 10mV. The upper table compares average values of normal azide response and b-wave amplitudes in rabbits with those obtained after poisoning with iodoacetic acid (IAA) and after administration of iodate. These are correlated with the average preservation of outer nuclear layer, pigment epithelium, and bipolar cells.

The latency of this reaction measures about four seconds and thus seems to be accounted for by the time necessary for the blood to carry the injected solution to the eye. The latent period is, moreover, unmeasurably short when the agent is injected into the common artery.

The reaction is also readily reversible as indicated by the fast return of the potential after its rapid rise. Indeed, rapid ascent of the potential and its fast decline appears to reflect arrival and passage of the column of blood containing a sample of the rapidly injected solution which becomes more diluted with further circulation.

Injection of high doses of azide or slow intravenous infusion increases the potential in a lasting manner. The potential thus seems to record the instantaneous concentration of azide in the blood. The threshold concentration of azide which produces a rise of the potential is very low, within the range of 10^{-6} molarity.

The reaction did not occur whenever the blood flow to the eye was blocked after the injection for a period of time at which normally the potential would have risen. On the other hand, removal of the anterior portion of the eye, including the ciliary body, did not abolish the reaction. The effect, therefore, can be defined as a rise of the steady potential across the retina.

According to the familiar concept that the steady potential originates from a differential polarization of the visual cells, one was inclined to assume a similar origin of this reaction. However, when the responsiveness to azide was tested in rabbits which lacked all visual abilities due to the administration of iodoacetic acid, the reaction was not found to be absent.

In 17 eyes in which the visual cell population had been destroyed almost completely by iodoacetic acid, 1.0 mg. azide, tested just previous to enucleation (several weeks after iodoacetic acid) produced an average response of 3.8 mV as compared to 10 mV in normal animals (see table of fig. 1). Moreover, there were several cases in this series of experiments in which the amplitude of the reaction almost reached the range obtained in the controls despite the virtually complete disappearance of the visual cells.

When, however, the same test was performed in rabbits poisoned by iodate (see table of fig. 1), the azide responsiveness had generally vanished, while reactions to illumination were less severely impaired than after iodoacetic acid. This greater preferential effect of iodate on azide responsiveness correlates with the more extensive destruction of the pigment epithelium by this poison as compared to iodoacetic acid. Moreover, the variations in the amplitude of the azide response after iodoacetic acid also significantly correlates with variations in the preservation of the pigment epithelium. On the other hand, no correlation between azide response and the preservation of any other retinal layer or membrane was found when the anatomic measurements (from camera lucida pictures) and the electrophysiologic data were evaluated. The experiments, therefore, establish that the pigment epithelium is at least one essential element of the electrical reaction to azide. Consequently, the pigment epithelium must also participate in the production of the normal direct current potential across the eye.

Origin of c-wave

This dependence of the azide response on the integrity of the pigment epithelium also provides an indication as to site of origin of certain components of the electroretinogram. In the experiment of Figure 2 (albino rabbit) the responsiveness to illumination and to azide is tested alternately at the same eye. The right column shows the response to azide, whereas the middle column records with the same amplifier settings the electroretinogram in response to a strong stimulus of short duration.

As it is typical for the rabbit, the c-wave is the most prominent component of the response in case a high stimulus intensity

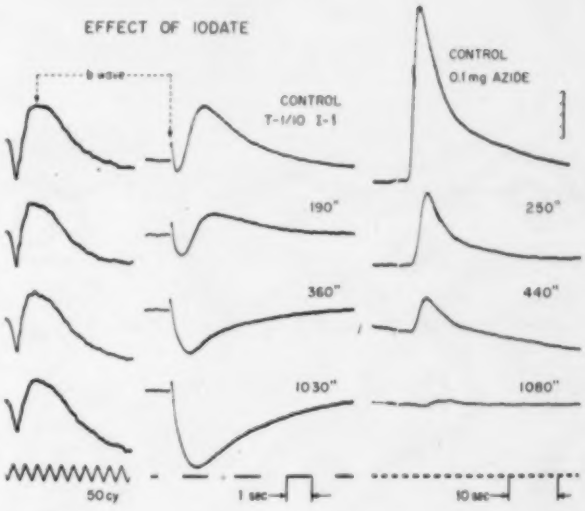

Fig. 2 (Noell). *Effect of iodate on c-wave and azide response.*

Rabbit, right eye; electrodes across globe, direct-current amplifier. Left column shows on single sweep the a- and b-waves of the electroretinograms of the middle column. The light stimulus has a duration of 130 milliseconds. The amplification of the left column is higher than in middle column. Right column presents the responses to 0.1 mg. NaN_3 in 2.0 cc., injected rapidly into leg vein; the interruption in the baseline marks the time of azide injection. Amplification is the same in middle and right columns, the time base differs. After the control tracings, 3.0 cc. of a five-percent iodate solution are administered intravenously. The numbers beside the tracings give the time in seconds after iodate. Vertical scale is 0.1 in mV units.

is employed. Because the initial portion of the electroretinogram is not well traced with the slow recording speed required for c-wave measurements, a second oscillograph records the a- and b-waves of the same response with higher amplification on a fast, stimulus triggered sweep.

The experiment measures the effect of iodate which is injected shortly after the control tracings have been obtained. There is a rapid effect upon the azide response which diminishes to less than half the control size within four minutes and has practically disappeared after 20 minutes. No significant changes in a- and b-waves are associated with this effect. However, a concomitant reduction in c-wave amplitude is evident and the c-wave completely disappears while a slow, polarity reversed potential develops upon which the c-wave seems to be superimposed normally.

The effect of iodate on both phenomena started in all experiments at about the same time and both reactions disappeared simultaneously. This was independent of the iodate doses employed and of the light stimuli tested as to their ability to produce a c-wave. Moreover, retinal excitation as measured by the optic tract potentials was always preserved when iodate had completely abolished c-wave and azide response. The latter finding, together with the preservation of a- and b-waves, is unequivocal in indicating that the c-wave is independent from electrical events of retinal excitation.

A relationship between azide response and c-wave is further indicated by a specific azide-sensitivity of the c-wave potential. In a similar manner as azide raises the level of the steady potential across the eye, azide increases the c-wave while other components of the electroretinogram are practically unchanged (fig. 3). Moreover, high doses of azide of systemic toxicity which depress the b-wave, still increase the c-wave beyond the effect produced by doses just ineffective in depressing the b-wave. The magnitude of the azide reaction is comparable to that of the direct-current potential change; on a percentage basis, the enhancement of the c-wave may even exceed the increase in direct-current level. The process, therefore, which generates the c-wave seems to be as specifically affected by azide as the reacting component of the steady potential.

Azide response and c-wave are, however,

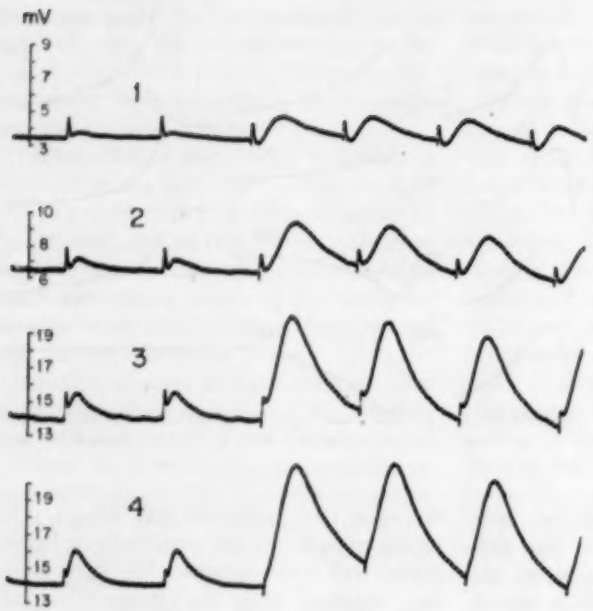

Fig. 3 (Noell). *Effect of azide on the a-, b-, and c-waves.*

Rabbit. The potential across the eye is recorded by direct-current amplifier. The eye is exposed to low intensity flashes (I-1/100), one every four seconds. During the illustrated periods, the intensity of the flash is transiently increased 100 times (the two left-sided responses are with I-1/100, the following with I-1). Azide is administered intermittently throughout the experiment, resulting in stepwise increases of the direct-current level. Tracing *1* is recorded after the direct-current potential has been increased by 1.3 mV from control value in response to 2.5 mg. NaN_3; tracing *2*—26 minutes after a total of 10 mg. has been injected since start of the experiment; *3*—59 minutes after *1* when the total amount of the injected azide has reached 55 mg.; *4*—17 minutes later after additional administration of 30 mg. in three injections. Note the additional increase of the c-wave in *4* while direct-current responsiveness has diminished and the b-wave has been reduced. The animal is anesthetized with urethane and artificially ventilated after immobilization by curare.

differently affected by iodoacetic acid. The azide reaction of the direct-current potential is not abolished when iodoacetic acid has destroyed the visual cells. All electrical reactions to illumination, however, have then disappeared and this includes the c-wave.

The explanation of azide response and c-wave origin rests upon a consideration of the electrophysiologic properties of epithelial structures. The pigment epithelium on which both phenomena evidently depend in a direct or indirect manner may be regarded as a boundary between two chemically different compartments, the strongly metabolizing retina on the one side and the richly vascularizing choroidea on the other.

In vertebrates, in which the retina itself is not vascularized, as for instance in the rabbit, practically the whole exchange of electrolytes and nonelectrolytes between blood and retina must occur across this boundary. In other vertebrates, as in carnivora and primates, the exchange across the pigment epithelium serves the requirements of the visual cell layers while the inner layers are supplied by the internal ophthalmic artery.

Differences in the concentration of ions at both sides of such a boundary and differences in permeability cause a potential difference on purely physicochemical grounds. In addition, such living boundaries are likely to participate actively in the transport of ions and thus may actively determine the development of electrical potentials. This, for instance, is well established for Na^+ transport across the frog's skin (Ussing, 1949).

Immersed in Ringer solution the isolated skin shows a high potential difference between inside and outside. This potential is caused by the active transport of Na^+ from the outside into the inside solution, and the

resulting electromotive forces constitute the measured potential difference. The basal layer of the skin epithelium which maintains the transport process is analogous to the pigment epithelium; the homogenous basal membrane of the frog's skin corresponds to the glass-membrane of the pigment epithelium. Since both the basal epithelium and the basal membrane of the skin have selective permeability, both corresponding structures of the retina should be considered as a possible source for the potential difference.

The action of azide on the potential difference across the eye is believed to result from the interference of this poison with active transport mechanisms across the pigment epithelium. The great rapidity of the azide action indicates that the poison must affect the very process which generates a potential across the eye. Moreover, the low concentration with which the agent becomes effective renders improbable the possibility that the agent alters physical properties of the epithelium which determine permeability. It rather seems as if azide interferes with an enzymatic component on which the active exchange of ions across the pigment epithelium depends.

Support for this assumption is given by experimental evidence on the interference of azide with the ion transport across the membranes of yeast cells, of the parietal cells (gastric mucosa) and of plant roots (Conway, 1949; Lundegardh, 1950).

As found by Conway, a marked accumulation of acid occurs outside fermenting yeast cells resulting from the exchange of outside potassium and inside hydrogen ions. Sodium azide inhibits this acid excretion in doses which are approximately the same as in our experiments. Moreover, the effect of azide on the yeast cell shows a similar rapidity and is similarly not produced by hydrocyanic acid.

On the basis of this analogy, azide is assumed to interfere directly with active ion transport across the pigment epithelium and to produce by such interference the observed electrical reaction. We have no evidence as to the kind of ions involved but the participation of hydrogen ions as in the yeast cell experiments may be suggested by the high glycolytic activity of the visual cells and by the essential role which the sulfhydryl group plays in the bleaching and synthesis of rhodopsin (Wald and Brown, 1952).

The great similarity between c-wave and azide appears sufficient to indicate that the same physicochemical system is involved in the generation of both potentials and that the c-wave represents a response to illumination of the same ion transport system with which azide directly interferes. Since the effect of iodoacetic acid on the visual cells abolishes all electrical reactions, including the c-wave, while the azide responsiveness survives, the initial event for the generation of the c-wave must be a change in the ion concentration at the retinal side of the pigment epithelium.

It is assumed that reactions within the visual cells lead to ionic changes in their environment and that these extracellular changes provide the stimulus for the pigment epithelium and provoke a change in its "membrane" activity (see schematic drawing of fig. 4). This particular "membrane" property of the pigment epithelium on which the c-wave depends, is defined by its azide sensitivity. Inasmuch as azide alters the responsiveness of the membrane during steady state conditions in producing a rise of the direct-current potential it also increases the responsiveness to "stimuli" originating from retinal illumination, that is, it re-enforces the c-wave.

A possible correlation between c-wave and pigment epithelium has occasionally been considered in the literature, generally with a negative conclusion, since illumination of the isolated pigment epithelium does not produce a potential change. Therman found that adrenalin rather specifically increases the c-wave of the frog's retina, apparently in a similar manner as azide in our experiments.

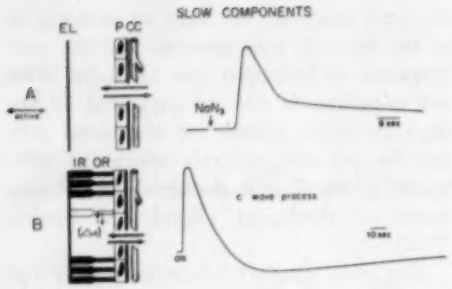

Fig. 4 (Noell). *Schematic representation of the possible origin of azide response (A) and c-wave (B).*

Both phenomena are assumed to depend upon a specific membrane property of the pigment epithelium resulting in active ion transport between retina and choroid. Photoreceptors and nervous elements do not directly participate in the generation of these potentials. The "stimulus" for the c-wave is a change in ionic concentration (Δc) resulting from rod stimulation. (EL) Outer limiting membrane. (P) Pigment epithelium. (CC) Choroid. (IR) Inner limb. (OR) Outer limb. The potential which represents the c-wave is computed by subtracting the cornea-negative potential (isolated by iodate) from the "normal" c-wave.

Since adrenalin also produces an advance of the pigment cell processes in the frog as illumination does after dark adaptation, the effect of adrenalin on the c-wave suggested a possible participation of pigment cell reactions in the generation of this potential.

Granit rather rejected this hypothesis on the ground that higher vertebrates, which lack pigment movements, nevertheless produce a c-wave. With respect to our analysis it is, however, of interest that adrenalin strongly affects the potential difference across the frog's skin and thus may have a similar action on the pigment epithelium.

In disavowing the possibility that the c-wave of the typical electroretinogram is caused by iris movements, Bruecke and Garten placed its origin at the visual cell level, while Granit assumed that it originates chiefly, if not exclusively, in the "rod pathway" thus implementing the participation of nervous structures. Both views are obviously not in agreement with the results of this analysis.

Origin of a-waves and b-waves

While the c-wave has been rather neglected in the past, the diphasic transient of the electroretinogram at the onset of illumination has been studied extensively. Since Kühne and Steiner, the diphasic response is considered to be the sum of two component potentials of opposite polarity.

Granit assumes that the cornea-positive component manifested by the b-wave originates "somewhere between the receptors and the ganglion cells"; whereas, the cornea-negative potential, normally indicated by the a-wave, is considered to originate from probably two reactions, one localized in the neural structures, the other in the receptors.

Since the a-wave is first to appear when strong illuminations are tested, Bartley assigns the a-wave to the receptors and the b-wave to the bipolar cells. This latter view has been accepted by Karpe for the clinical interpretation of electroretinograms.

Recently, Ottoson and Svaetichin concluded from microelectrode studies of the frog's retina that the electroretinogram is generated exclusively by the receptor layer, while Tomita and Funaishi in a similar study did not exclude the nervous layers as the elements for a- and b-wave generation.

Studies on the origin of a- and b-waves and their association with other phenomena of retinal excitation have been much stimulated in the past by the selective effects of chemicals upon one or the other potential. However, since the site of action of these chemicals was unknown no definite conclusion as to the location of the potentials they isolated were obtained in such experiments. A distinct analytic advantage must, therefore, be ascribed to the action of iodoacetic acid and iodate because of their histologically verified site of action.

Since iodoacetic acid selectively affects the viability of the visual cells, its immediate action on electrical phenomena of the eye must result from visual cell impairment. Accordingly, any selective effects the poison may have must be explained on such basis.

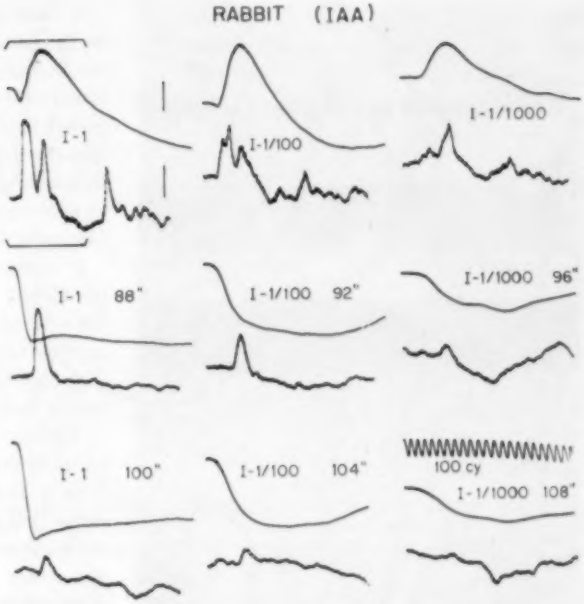

Fig. 5 (Noell). *Effect of iodoacetic acid on initial portion of the electroretinogram and optic tract potential.*

Rabbit. Electroretinogram and optic tract potential are recorded with double-beam oscillograph. The upper beam records a- and b-waves, the lower beam traces the optic tract potential. The beams are triggered in common by the light flash, the duration of which is marked on top and bottom of the first record. The stimulus is tungsten light. (I) Indicates intensity in arbitrary units. Upper row illustrates records during the control period; the lower rows after intravenous administration of 20 mg./kg. iodoacetic acid. The numbers beside the tracings (for example 88") give the time in seconds after the start of injection.

In rabbits, the effect of iodoacetic acid on retinal excitation develops with a characteristic sequence (fig. 5). The poison abolishes first the b-wave while its effect upon the a-wave and the slow potentials (for instance, the c-wave) develops with some delay. In accordance with a well-known result of Granit's extensive experiments, that the b-wave process is closely linked with the excitation of the retinal pathway, there is a concurrent reduction of the optic tract potentials when the b-wave disappears.

Two details of this effect are particularly noteworthy from an analytic point of view:

1. At the time the optic tract potentials have disappeared, the a-wave potential is obviously not impaired; on the contrary the isolated a-wave potential is of amplitudes as have rarely been found with other experimental procedures (see third row of fig. 5).

2. The rate of b-wave elimination is faster than the rate of reduction in optic tract potentials. At times after the injection of iodoacetic acid at which the b-wave has markedly declined, impulses in response to illumination are still initiated and reach the optic tract where they give rise to a typical compound potential (see second row of fig. 5).

Since the a-wave potential in these experiments is not impaired at the time retinal excitation has been blocked, all layers vitread the visual cells are excluded as a possible site of a-wave generation. The potential must originate in front of the block and since this block is established somewhere at the visual cell level, according to the primary site of action of iodoacetic acid, even the most proximal portions of the visual cells cannot be assumed to participate in the generation of this potential. The potential must develop posterior to at least the outer nuclear layer.

On the other hand, the rapid disappearance of the b-wave with the action of iodoacetic acid on the visual cells indicates either the direct participation of the visual cells in the generation of the b-wave or their indirect participation by initiating excitatory processes vitread the visual cells which in turn are directly responsible for the poten-

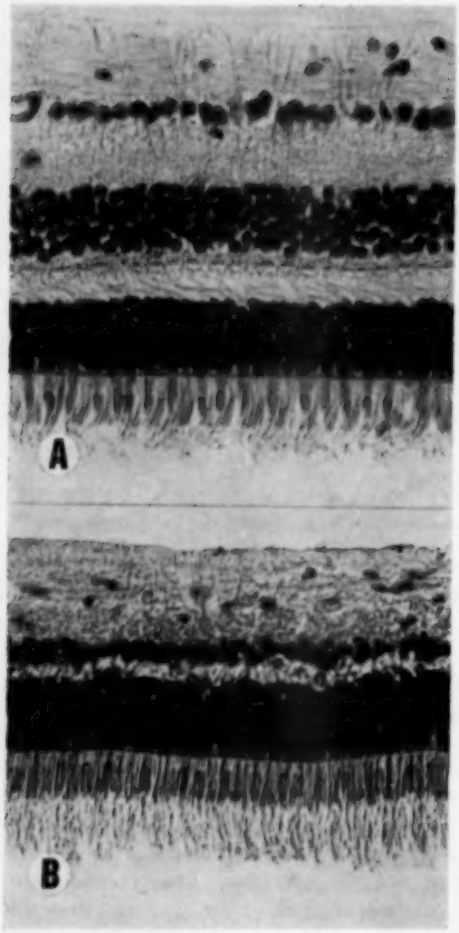

Fig. 6 (Noell). *Retina from rhesus monkey.* Three weeks after transient interruption of retinal blood flow (B) compared with normal control (A). (Top) Hematoxylin-phloxine (×350). (Bottom) Hematoxylin-eosin (×350).

tial. However, since the b-wave practically disappears before the propagation of excitation through the retina has been blocked, the iodoacetic acid experiment does not support the assumptions of the literature that the b-wave is indicative of the passage of excitation along the bipolar cells. The b-wave process must be much more closely linked to functional processes of the visual cells than such an hypothesis suggests.

A close dependence of the b-wave process on the integrity of the proximal portions of the visual cells and on the linkage of the visual cells with the nervous pathway is suggested by the well supported clinical finding that the impairment of retinal blood flow by embolism or central vein thrombosis results in subnormal or negative electroretinograms (Bounds, 1953).

I simulated this pathologic process by transiently blocking the blood flow through the central artery in monkeys, carefully preventing any change of the choroidal system. Figure 6 illustrates sections through such an eye in comparison to a normal one.

The eye was enucleated three weeks after surgical exposure of the optic nerve at which time a fine cut through the center of the nerve had resulted in the immediate collapse of the retinal arteries and the blanching of the whole retina, followed by the development of retinal edema.

Three days after surgery the edema had subsided and retinal circulation appeared to be restored for unknown reasons. The inner retinal layers of the eye were nevertheless extensively damaged except for the most anterior portion of the retina at distances from the ora serrata of about three to five mm. With the exception of this region, the ganglion cells and optic nerve fibers had completely degenerated and the inner nuclear layer was reduced to about one row, while the outer layers of the retina did not reveal pathology. However, despite the preservation of the visual cells, the electroretinogram of the eye, tested just prior to enucleation, had distinctly suffered (fig. 7).

In comparison to the undamaged contralateral eye, the b-wave is markedly reduced and the a-wave more prominent but less rapidly developing. This is taken to indicate that the integrity of an isolated visual cell layer and of its nutritional system is alone not sufficient to maintain the generation of a typical b-wave and an entirely normal a-wave. If these potentials, as suggested by the preceding discussion, are closely linked

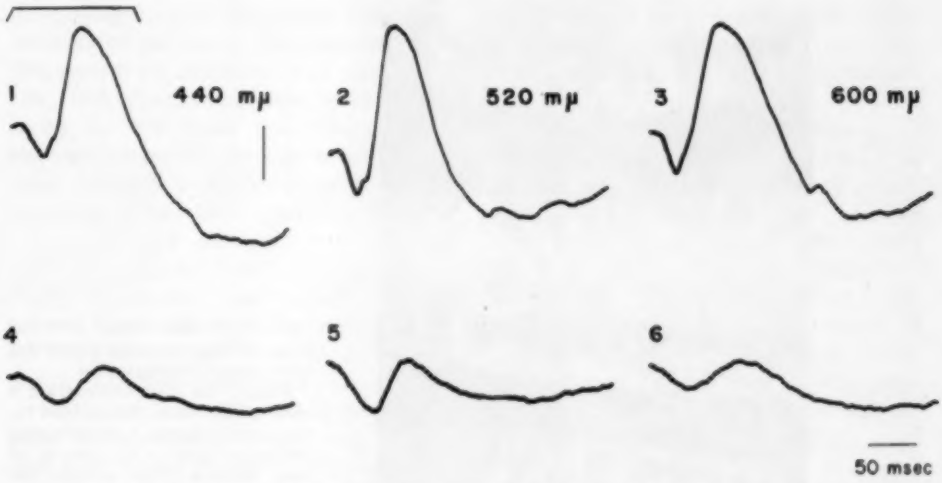

Fig. 7 (Noell). *Electroretinograms from rhesus monkey.* (Lower row) three weeks after transient interruption of retinal blood flow (same eye as in Figure 6, B). Upper row—controls from contralateral, undamaged eye recorded simultaneously with lower row.

to electrical processes within the visual cell layer, functions of the adjacent nervous elements may be an equally important factor of their generation; at least we must state that the electrical function of the visual cell layer is affected when the adjacent nerve cells have suffered.

By providing a correlation of various degrees of electrical changes with histologic effects, the action of iodate enables a further clarification as to the participation of the visual cells in the generation of a- and b-waves. Several days generally pass until the a- and b-waves disappear after the injection of iodate in contrast to the immediate action of the poison on the pigment epithelium as measured by the azide responsiveness. The progress of these a- and b-wave changes was followed by daily recordings and the eyes removed at various stages of the effect.

Typical sections of two retinas obtained from different rabbits at the fourth day after the injection (five cc. of two-percent solution) are illustrated in Figure 8 together with the electroretinograms obtained prior to enucleation.

Both electroretinograms differ from their controls, recorded before the injection, by an increase in latent period and by a reduction in the amplitude of the a-wave. In the one case (A) the a-wave has almost disappeared; in the other, a slowing of the a-wave probably can account for the apparent amplitude reduction. The b-wave is of almost the same amplitude as before the injection in both cases.

The microphotographs, however, reveal that at the time these electroretinograms were recorded, the outer limbs of the visual cells had been affected extensively. The outer limbs have either deteriorated (see A) or they form shrunken fibers (see B) still attached to the inner limbs. The latter have also suffered; the individual inner limb is less distinctly stained than normal but the continuity of the inner limb layer is generally preserved. The pigment epithelium (not illustrated) was completely destroyed in both cases.

This surprising discrepancy between the severity of damage of the rods and the maintenance of electrical reaction makes it unlikely that the outer limbs directly contribute to the generation of a- and b-waves. The experi-

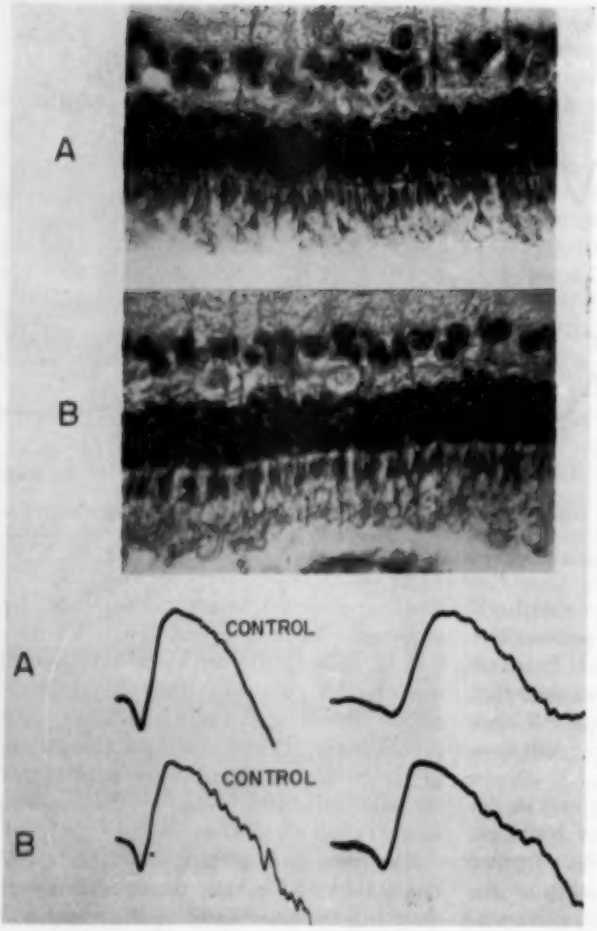

Fig. 8 (Noell). *Visual cells and electroretinogram on the fourth day after iodate administration.*

Rabbit. The electroretinogram is recorded by cotton thread from the cocainized cornea. Amplification and sweep speed is the same in all four records. The sections are stained with hematoxylin-eosin (×700). The right-handed electroretinograms were recorded just prior to enucleation and Zenker fixation of the unopened globes.

ment excludes the outer limbs as the site of the b-wave potential. It also renders improbable the possibility that the a-wave originates from differential depolarization of the free and basal ends of the outer limbs as suggested by the hypothesis concerning the steady potential across the eye.

The outer-limb changes in retina B of Figure 8 are of such severity that the a-wave recorded in this experiment prior to enucleation can hardly result from current flow between the two ends of the outer limb. The experiment rather suggests that the a-wave is initiated by reactions which occur close to the linkage between outer and inner limb.

Micro-electrode studies of the potentials inside the rabbit's retina generally supported the results of the preceding experiments. They supplied further evidence that a- and b-waves are different manifestations of retinal activity. Moreover, these studies indicated that the a-wave potential has a different origin than the slow potential of cornea-negative polarity which develops when iodate has removed the c-wave (see fig. 2).

This slow cornea-negative potential which takes the place of the c-wave is interpreted to represent the "passive" component of the

ionic changes which occur in the outer compartment of the retina. The potential probably records the development of ionic gradients, and their dissipation by diffusion, across the boundaries (for example, pigment epithelium) of the outer layers. The a-wave potential, however, seems to be the compound of individual visual cell reactions.

In assigning a- and b-waves to electrical reactions associated with the activity of excitable structures, consideration must be given to the fact that the electroretinogram is not recorded directly from the retina but that it manifests itself as a current flow across the eye. The individual element of the retina is embedded in an extensive volume conductor. Action potentials which such elements may produce, due to a slow propagation of excitation or due to a different reactivity of the two ends of the element, are measurable only in the very vicinity of the element for the simple reason that source and sink of the "action" current are very close together.

More extensive spread of such current does, however, result when the flow of current parallel to the element is hindered by a close packing of the element with others of the same kind and same orientation which undergo simultaneously the same electrical change.

It follows that all retinal elements which do not satisfy this condition cannot contribute an "action potential" to the electroretinogram. This applies, for instance, to the retinal ganglion cells; their electrical activity escapes detection unless the electrode is placed directly upon the inner surface of the retina; accordingly ganglion cell degeneration after severance of the optic nerve does not affect the electroretinogram as was measured in this laboratory and as it is well known in clinical electroretinography.

Our analysis limits the possible sites of a- and b-wave generation to a region extending from outer limbs to (and including) the outer plexiform layer. Among the structures of this region, outer and inner limbs best satisfy the condition necessary in the described manner to produce current flow across the retina which extends to the cornea.

The discrepancy between maintenance of a- and b-wave and structural changes of the rods, as observed in the iodate experiments, places preference upon the most closely packed inner limbs as it was almost 100 years ago suggested by Kühne and Steiner. If so, the inner limbs must be regarded as but one ("electronic") element of a- and b-waves. In case of the a-wave, current flow would probably not develop without specific reaction at the linkage between inner and outer limb, and similarly a b-wave current probably does not ensue unless structures proximal the inner limbs participate in the excitation process.

The interpretation of the electroretinogram in terms of current flow across the eye applies particularly to problems of clinical electroretinography.

It readily explains the fact that in advanced cases of retinitis pigmentosa no electrical manifestation of the preserved central field is recorded since the current flow is restricted to too small an area to extend measurably to the cornea.

It also offers an explanation to the fact that the isolated cone function in early cases of retinitis pigmentosa may not be associated with electroretinographic manifestations since any field of current is likely to be shunted through the inactive or degenerated rods.

Furthermore it reveals that isolated visual cell function in the periphery close to the ora serrata is more likely to be associated with a somewhat preserved electroretinogram than a similar large field in central regions since current flow originating in the periphery has a greater chance to extend measurably to the site of the electrode.

Summary

1. A brief account is given of experiments designed to analyze the origin of the electrical

potentials across the eye, especially those in response to illumination (electroretinogram). 2. By means of the selective effects of agents upon visual cells and pigment epithelium, it is shown that the c-wave represents a reaction of the potential difference across the pigment epithelium in response to ionic concentration changes produced by the visual cells. The membrane property of the pigment epithelium responsible for the c-wave is identified by its sensitivity to sodium azide.

3. By excluding other possibilities, the row of inner limbs is shown to represent one essential element for the generation of a-waves and b-waves, but the area involved in the production of a-waves and b-waves seems to extend from the region of the outer limbs to (and including) the outer plexiform layer.

School of Aviation Medicine.

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GENERAL DISCUSSION ON THE ELECTRORETINOGRAM

CHAIRMAN MCDONALD: We have with us this afternoon a distinguished visitor from Geneva, Switzerland, Dr. Franceschetti. I would like to ask him if he would care to discuss the papers that have been presented so far.

DR. A. FRANCESCHETTI (Geneva, Switzerland): I thank you for this opportunity to say a few words about retinograms. I would especially emphasize the fact that the findings of Dr. Riggs, explaining the results in retinitis pigmentosa, are not only extremely good from a theoretical standpoint, but especially also from a practical standpoint.

For a few years we have made examinations of retinograms in patients with retinal diseases, but today I shall speak only about the question of retinitis pigmentosa and especially of the differential diagnosis, which seems to me most important.

As a matter of fact, we have had exactly the same results as those of Karpe and Riggs, and also in cases of congenital hemianopia—no response. I think that is very important in the diagnosis of retinitis pigmentosa.

What I would emphasize is the question of differential diagnosis with pseudoretinitis pigmentosa. The alteration of the fundus resembles retinitis pigmentosa, but yet is not retinitis pigmentosa, but is due to general infectious disease.

I would like to show you the slides of two cases which I think show nicely the importance, from

the clinical standpoint, of the diagnosis by the retinogram.

(Slide). You see on the right side of this slide a normal electroretinogram. Here is the stimulus, and here is the normal response of the electroretinogram in a normal case.

Here we have a case of a woman who is now about 60 years of age. About 20 years ago she began to lose the vision in her left eye. Clinically it is a real pseudoretinitis pigmentosa, with a narrow vessel with pigmentation. It may be a little larger than in real retinitis pigmentosa, but nevertheless clinically it is similar.

You see here there is no response to the electroretinogram. So, the first thing we see in the final stage are alterations of the fundus where it is in a moment of fulguration. We are not able to say if it is primary retinitis pigmentosa or if it is a complete atrophy of the retina.

What is important, however, is the other eye. In the other eye she also has a pseudoretinitis pigmentosa, but fairly good vision. Nevertheless, in the last 10 years the vision in the peripheral visual field was diminished.

Here you see the reaction to the electroretinogram. You see it is really subnormal if you compare it with the normal. The response is less, but nevertheless we can say from this result that it cannot be a real retinitis pigmentosa, because, as you have heard today, even in cases with fairly good adaptation and good central region adaptation, in the very beginning of such cases we get practically no response.

So, in this case, the fundus looking like retinitis pigmentosa on the left side, one would be unable to see anything because there is complete involvement of the retina.

It makes a certain difference, also, from the point of view of prognosis. In this case, as we found out later, the patient had been treated for syphilis about 30 years ago, with negative reactions now. After seeing the nearly good reaction of the electroretinogram and the response, we can say this is something that has filled with pigmentation. In this case we can say the prognosis is rather good.

(Slide). This second case is also interesting, not only from a clinical point of view but also because there were certain genetic problems in it. This woman is now 57 years of age. I saw her for the first time in 1939. At that time she presented retinitis pigmentosa in her right eye, and a normal left eye.

When retinitis pigmentosa exists in only one eye, the question always is, "How about the other eye?" In general, this case has been seen for 10 or more years, so I think I can confirm the fact that this is a lateral retinitis pigmentosa. Today, with the retinogram, we can confirm the fact much more easily and quickly, because always we say we must wait 10, 15, or 20 years, and after that time we should find the typical alteration.

In this slide you see that before we made the studies on the electroretinogram, about four years ago, we found this to be lateral pigmentosa. You see no response here on this side, but she had no bad central vision.

I check this patient once a year. Last year in the periphery of the left eye I saw the beginning of atrophia choroideae et retinae. A few years ago I published a case of atrophia gyratus, and I observed it in families with retinitis pigmentosa.

And so we see on this side a pigmentosa, and on the other side we see atrophia gyratus. I have seen retinitis pigmentosa develop in the left eye, and in the right eye I saw the adaptation was very bad, and the visual field was equally bad.

The interesting thing is the response to electroretinography, which is quite good. Here is a normal eye. This is subnormal, but nevertheless quite good. That confirms that there exists unilateral retinitis pigmentosa, as we now know, and even early retinitis pigmentosa gives no response.

This is also interesting from a genetic point of view—that in one eye there is retinitis pigmentosa, and in the other eye late atrophia gyratus choroideae et retinae, which we know may be present in the same genealogic tree but which is extraordinary to find in the same person; and we can also say today that we cannot speak of the beginning of the same affection, otherwise we would have no response.

From the clinical standpoint, we must consider the prognosis as relatively good, as we have a nearly good response; and so we can say this confirms that it is unilateral retinitis pigmentosa, that the prognosis is not bad, and that the electroretinogram is almost good.

Dr. Frank Carroll (New York): Recently (Arch. Ophth., **53**:35 (July) 1953), Dr. Haig and I reported a family with congenital stationery night blindness without ophthalmoscopic or other abnormalities. These individuals have normal cone function, but no rod function. We thus had a most unusual opportunity to study cone function in the human being. Dr. Riggs was good enough to perform electroretinography on two of these persons and his results are, I believe, very important in our understanding of the normal, as well as the abnormal electroretinogram. He found that if there was no rod function there was no b-wave; since these individuals had normal cone function, they had a normal a-wave. In retinitis pigmentosa there is no a- or b-wave. For the first time, as far as I know, Dr. Riggs has shown how we can distinguish by electroretinography between retinitis pigmentosa and congenital stationary night blindness without ophthalmoscopic or other abnormalities. This is a significant contribution.

Since this is the first time, to my knowledge, that human beings with normal cone function and no rod function have been carefully studied by electroretinography, it is the first time that we are sure that in human beings the a-wave is concerned with cone function and the b-wave with rod function—a very significant contribution.

Dr. Hermann M. Burian (Iowa City, Iowa): In commenting on the papers presented this after-

noon, I should like to make a few remarks and speak first about the point made by Dr. Riggs that light scatter makes it very difficult to stimulate electively different parts of the retina.

Being well aware of this we are at the present time engaged in trying to use X rays instead of light as stimulus in order to prevent scatter and to achieve pointlike stimulation. X rays have been used in animal experimentation and we hope that our human experiments will also be successful.

The problem raised by both Dr. Riggs and Dr. Noell, namely that vision may persist while the electroretinogram is extinguished, or vice versa, may have various explanations. We are particularly interested in the possibility that this behavior may permit us to distinguish between affections of the retina and affections of the optic nerve.

The question arises not too infrequently whether we are faced with a retrobulbar neuritis or a retinal affection, and we have been able in a number of cases to decide that the loss of vision was unlikely to be of retinal origin, because of the fact that the electroretinogram was normal.

In patients with absolute glaucoma—our last patient was completely blind for two years—we have also obtained normal electroretinograms. This is, of course, of extreme interest with regard to the origin of the electrical potentials in the retina, and I should have been very happy if I could have had the eye of our last patient for histologic investigation so as to obtain slides from humans comparable to those which Dr. Noell has shown us today. However, fortunately for him and unfortunately for us, this patient had no pain or other complaint concerning his eye. In any case, from what Dr. Noell has shown and from what we know of the pathologic anatomy of glaucoma, it would seem that the ganglion cells do not contribute to the formation of the electroretinogram.

I am particularly grateful that Dr. Noell has emphasized the importance of the different elements of the electroretinogram—the a-wave, the b-wave, and the c-wave. We are inclined to rely too much on the b-wave and tend to forget the other parts of the electroretinogram. I believe that they can give us considerable information also from a clinical standpoint.

As an example, I should like to quote the fact that we have seen, in patients with detached retina who had a very subnormal electroretinogram, after successful operation a remarkable increase in the a-wave while the b-wave remained subnormal, probably indicating a return of function of the sensory epithelium.

Finally I should like to mention a phenomenon which I have observed and which puzzles me very much. Maybe Dr. Riggs or Dr. Noell has an idea as to what it may be related.

We are stimulating the retina with very high intensity stroboscopic light stimuli. We light-adapt our patients or experimental subjects, and then we dark-adapt them for 30 or 40 minutes. Then, when we turn on the room light again to light-adapt them, we obtain a very subnormal—in fact, at first a negative—electroretinogram. It takes from six to 20 minutes before the electroretinogram regains the size it had prior to the dark adaptation. If the illumination is considerably increased right after the dark adaptation, the return to normal is more rapid.

Dr. George W. Bounds, Jr. (Iowa City, Iowa): Dr. Burian and I have been studying one family with choroideremia. There are two male members and four female members in the family. One male member has a very far-advanced case, with his central vision down now to about 20/100, and a marked constriction of his visual field. He has an extinguished electroretinogram. Although his brother has much better acuity and visual fields, he also has an extinguished electroretinogram.

The female members of the family all have the typical pigment that has been described in the retina, but their central vision in all cases is good. However, they have a normal electroretinogram, but each one of them presents some variation. We consider these within normal range.

Dr. Lorrin A. Riggs (Providence, Rhode Island): Dr. Burian asked for a comment on his observation about the repeated light adaptation after dark adaptation. I think this is an extremely interesting finding. I don't recall seeing it described in the literature before.

We have found, much to our embarrassment in studies of dark adaptation, the same thing to be more or less true in dark adaptation studies, namely, that the course of dark adaptation initially can be followed very readily. Then one light-adapts and tries to get further data by a second run of dark adaptation after a fairly bright light-adaptive field. Usually the thing will not track at all. The second course of dark adaptation is usually more rapid, and it does not follow quite right in terms of the first dark adaptation.

I think very likely the explanation lies in research, such as Dr. Wall has been conducting, and I would suggest that the processes involved here are not a single process of regeneration and dark adaptation and breaking down during light adaptation, but rather multiple processes, perhaps a visual cycle such as he originally described; and consequently I believe very likely the time relations are very critical as to which part of the cycle is affected—the initial light adaptation, which is presumably longer and more complete, and the second light adaptation, which is shorter because it is done in the laboratory in a limited amount of time.

Dr. W. K. Noell (Randolph Field, Texas): Monkeys treated by iodoacetate develop the same retinal changes as in retinitis pigmentosa with preservation of the cones in the central area. In these animals a small b-wave is recorded whenever the visual cells close to the ora serrata have survived for distances of from three to five mm. This and other evidence supports the view that the periphery of the visual field does contribute more to the electroretinogram than central areas. The periphery of the retina is closer to our electrodes and the flow of current reaches the cornea much more effectively

than from central fields.

Subnormal b-waves with prominence of the a-wave were recorded in dark adapted rabbits which had been poisoned by relatively small doses of iodoacetate. During subsequent light adaptation the b-wave increased in size to almost normal values. We first thought that this was a significant observation for the iodoacetate effect but when we tested the same states of adaptation in normal animals we found that there was the same increase of the b-wave in response to light adaptation. Probably these subnormal b-wave ERGs are caused by a super-maximal a-wave which opposes the b-wave appearance in our records.

CHAIRMAN MCDONALD: I am sure we all appreciate the significance of these papers, in which there has been an objective measurement of the visual response. The apparatus that Dr. Wagman designed and is using I know can be used for many other experiments. It is something that no one has brought up today—that as a result of his experiments so far, the human is not much better than the grasshopper, as far as dark adaptation is concerned.

DETECTION OF INTRAOCULAR TUMORS BY THE USE OF RADIOACTIVE PHOSPHORUS*

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INTRODUCTION

Histologic examination for the diagnosis of malignant tissue is at present the most satisfactory means of making a positive diagnosis. In the case of intraocular tumors, however, excepting those situated on the iris, histologic confirmation is possible only after enucleation. In initiating the present study, therefore, it was considered important to utilize the selective localization of certain radioactive isotopes in tumor tissue to ascertain whether this might be a practical aid in the diagnosis of such intraocular tumors in vivo. This procedure is not intended to replace present methods of examination of intraocular lesions, but is presented rather as a valuable diagnostic aid.

It was known from earlier studies[1-4] that various tagged materials are selectively taken up by rapidly proliferating tissue and that this increased radioactivity can be detected in vivo by special counting procedures. Our investigation on the application to the problem of identification of intraocular tumors was a logical extension of this general method.

The particular clinical problem concerned is the differentiation between fluid detachment of the retina and detachments resulting from underlying malignant tumors. Although it often is possible to differentiate between these conditions by means of the usual diagnostic procedures (transillumination) and established criteria (presence of a retinal tear), many times such means are inadequate. It is in these doubtful cases that the present test has proved most valuable.

Since intraocular tumors are known to be adjacent to the outer surface of the eye, it is possible to use a radioactive substance which emits beta rays for their identification. The advantage in using beta rays for this purpose is that the measurement obtained by counting directly over the suspected tumor is essentially unaffected by radioactivity in the surrounding normal tissue, and thus the masking that might occur with a gamma-ray emitter is avoided.

P^{32} was the isotope chosen because other investigators had shown[1,4] that it is taken up in tumor tissue in considerably higher

* From the Department of Surgery, Division of Ophthalmology, and the Department of Radiology, Western Reserve University School of Medicine and University Hospitals. Research performed in part under AEC contract No. W-31-109-eng-78.

concentrations than in normal tissue. It emits beta particles which have adequate penetration in tissue (a maximum of eight mm.) and has a half-life sufficiently long (14.3 days) to allow ease of handling and scheduling of patients.

Method

The method used at the present time is essentially the same as that reported by the authors in an earlier paper,[5] but it has undergone some modification as to instruments and technique.

Counting apparatus

In the first report of this investigation, a small end-window Geiger counter* was used to record the measurements over points anterior to the equator of the eye and points slightly posterior to the equator. More recently, this has been replaced by a side-window Geiger counter,† having an angle of approximately 45 degrees (fig. 1). This enables the examiner to reach areas for counting much posterior to those obtained with the end-window counter. Preliminary work has also been done on the development of a scintillation counter which would allow counting directly over areas on the posterior half of the globe.

* Model 222, made by Anton Electronic Laboratory, Inc., 1226-1238 Flushing Avenue, Brooklyn, New York.

† Model 224, made by Anton Electronic Laboratory, Inc., 1226-1238 Flushing Avenue, Brooklyn, New York.

In vivo counting procedure

1. The patient is prepared by instilling one-percent tetracaine hydrochloride or four-percent cocaine hydrochloride into the conjunctival sac.

2. A thorough ophthalmoscopic examination is performed to determine the exact situation and extent of the suspected tumor.

3. After a check has been made to determine that the counting apparatus is operating properly, the radioactive phosphorus (P^{32}), 500 microcuries in sterile isotonic sodium chloride solution, is injected intravenously. This tracer dose of 500 microcuries is far below the level of radioactivity which might produce manifest injurious effects, and is less than that received during most diagnostic roentgenographic procedures. It is, however, greater than the generally accepted "permissible dose."

4. Immediately after the injection of the radioactive solution, the counting procedure is started by placing the counting tube or crystal in direct contact with the eye immediately adjacent to the suspected tumor (fig. 2). Measurement is made for one-half to one minute in this location and the count recorded. The counter is then moved to an unaffected portion of the eye, as far removed from the tumor site as possible, and a similar count is taken. This procedure is continued by alternating the counting between the suspected tumor site and the normal tissue for approximately 15 minutes.

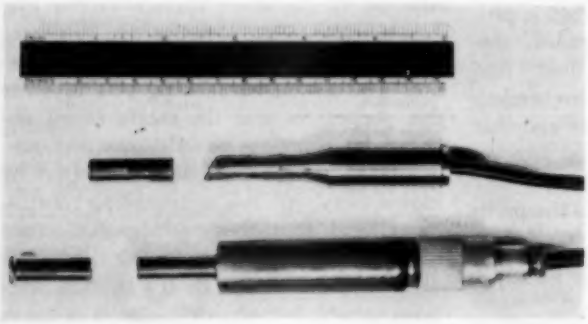

Fig. 1 (Thomas, et al.). Geiger counter used for eye measurements. (Manufactured by Anton Electronic Laboratory, Inc., 1226-38 Flushing Avenue, Brooklyn 6, New York).

Fig. 2 (Thomas, et al.). Use of Geiger counter on the living eye.

Comparative counts are then repeated one-half hour and one hour after the injection of radioactive phosphorus.

5. The variation in counts for the tumor and for the normal tissue, with the time of the readings, is plotted on ordinary graph paper. The ratio of tumor to normal counts as determined for the various times is recorded.

The present test differs from that used in the earlier tumor studies with P^{32} in that measurements are made within a very short time after injection of the radioactive material, rather than two to four days later. This change in technique was instituted as the result of preliminary studies on the early accumulation rate of P^{32} in neoplastic and in normal tissue.

Several patients with superficial malignant tumors were studied by using the following procedure:

Immediately after the injection of a 500-microcurie tracer dose of P^{32}; continuous recordings of counts over the tumor and over normal tissue were made for a period of one or two hours. Additional counts were taken the following day and at subsequent intervals for three or four days.

From these data, a graph showing the composite variation of the counts with time was prepared (fig. 3). This curve shows that the ratio of tumor counts to normal counts reaches a maximum very early (less than 15 minutes, for the patients studied) and then decreases for one or two hours following the injection. After this preliminary decrease, a subsequent increase in the ratio becomes evident and continues throughout the periods studied, probably reaching equilibrium in four or five days.

The cause for the accumulation of radio-

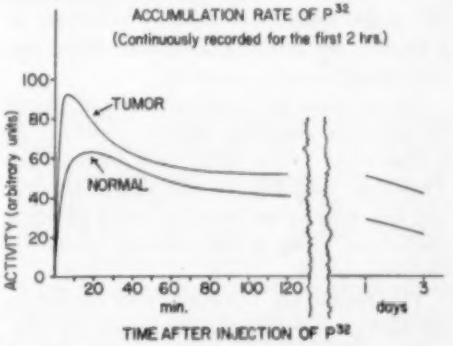

Fig. 3 (Thomas, et al.). Graph showing variation of counts with time interval.

activity in the tumor tissue immediately following the injection is not definitely known, and further investigations are being carried out in an effort to elucidate this phenomenon. It is thought at present that it may be due to one or all of the following: (1) Increased vascularity of the tumor, as compared with normal tissue; (2) more rapid transport of the inorganic phosphorus across the vascular membrane into the extracellular space; (3) more rapid transport of the phosphorus across the cellular membrane into the intracellular space; (4) more rapid incorporation of phosphorus into the nuclear and other cellular constituents.

In vitro counting procedure

In cases in which the eye is removed, if enucleation is carried out within four days after the injection of the tracer substance, no additional P^{32} is administered. If the period between the test for phosphorus uptake and enucleation is longer, another tracer dose of P^{32} is injected before operation.

As soon as possible after enucleation, the eye is sectioned directly through the tumor. One half of the specimen is used for the preparation of histologic sections and radioautographs. From the other half of the eye, samples of tumor, sclera, retina, and lens are obtained for assay of the radioactivity present in the various ocular tissues. The counts must be made on fresh specimens, for if the tissues are allowed to remain in a fixative for any length of time, there is a loss of activity by leaching.

The aliquots are weighed wet and placed in porcelain counting dishes, dissolved in concentrated nitric acid, and then dried. They thus yield thin flat samples. After drying, the samples are counted under identical conditions, using a conventional laboratory Geiger counter.

The results are recorded, with correction for background radiation, in counts per minute per gram of sample. The ratio of these values for tumor to normal tissue is then determined.

Interpretation of test

In the critical evaluation of the results, the lesions have been separated according to their position in the eye. In the case of a pathologic process in the anterior segment, the counter may be very closely apposed to the suspected area. In posterior sites, apposition is much more difficult and, in some cases, uncertain. With our present method, an elevated ratio in suspected tumors in the posterior segment may be significant, but a low ratio may mean only that the measurement is unsatisfactory because of unsuccessful apposition of the counter to the lesion.

Our studies so far indicate that a lesion in the anterior segment displaying a selective uptake ratio of 1.4 or higher is very strongly suggestive of tumor. A selective uptake ratio of less than 1.2 over the anterior segment is indicative of a non-neoplastic process. Despite the technical difficulty in counting measurements over posterior sites, a ratio exceeding 1.2 in this region appears to be significantly suggestive of neoplasm. The procedure of counting the radioactivity of posterior tumors is being greatly facilitated by the new type of angle-window counter.

In the interpretation of the results obtained with the present method, an additional problem arises as the result of the relatively low levels of radioactivity which are being measured. With the dosage of 500 microcuries of P^{32}, the counts are usually in the vicinity of 300 to 500 per minute. Since the patients experience considerable discomfort with long periods of measurement, the counting time has to be limited, as a rule, to approximately one minute or less. Hence it is obvious that the statistical accuracy is relatively poor for a single measurement and, for this reason, it is imperative that many measurements be made over both the tumor and the normal tissue in order to obtain valid results. Only by doing this is it possible to obtain results which are valid.

TEST RESULTS FOR CASES WITH PROVEN* DIAGNOSES

Case No.	Date	Ratio of Tumor to Normal c/min In Vivo	In Vitro	Disposition & Remarks	Pathological Findings (P) or Clinical Diagnosis (C)
			ANTERIOR TUMORS		
3	2-51	2.30	5.62	Enucleation for tumor	Melanoma of Choroid (P)
7	7-51	2.26	3.49	Enucleation for tumor	Melanoma of Choroid (P)
22	6-52	1.94	6.85	Enucleation for tumor	Melanosarcoma Choroid (P)
38	3-53	1.79	1.91	Enucleation for tumor	Melanoma of Choroid (P)
27	7-52	1.74	6.23	Enucleation for tumor	Melanoma of Choroid (P)
4	3-51	1.66	4.68	Enucleation for tumor	Melanosarcoma Choroid (P)
5	3-51	1.65	4.89	Enucleation for tumor	Melanosarcoma Choroid (P)
2	1-51	1.50	No Spec.	Enucleation for tumor	Melanoblastoma Choroid (P)
33	11-52	1.47	-	No procedure	Metastatic Ca (Breast)(C)
9	9-51	1.42	-	No procedure	Metastatic Ca (Breast)(C)
35	1-53	1.41	1.90	Enucleation for tumor	Melanoma of Choroid (P)
			POSTERIOR TUMOR		
14	2-52	1.27	14.25	Enucleation for tumor	Melanoma of Choroid (P)
18	5-52	1.24	No Spec.	Enucleation for tumor	Spindle-cell Ca (P)
17	5-52	1.21	3.82	Enucleation for tumor	Melanosarcoma Choroid (P)
			RETINOBLASTOMA		
37	2-53	0.95	5.40	Enucleation for tumor	Retinoblastoma (P)
43	4-53	1.15	-	X-Ray therapy	Retinoblastoma in second eye, first eye enucleated (P)
			NON-TUMORS		
15	4-52	1.31	-	Enucleated for Uveitis	Uveitis (P)
34	12-52	1.23	-	No procedure	Uveitis (C)
26	7-52	1.20	-	Operated for hemorrhage	Proven hemorrhage (P)
6	5-51	1.20	-	Reattached	Fluid detachment (C)
40	4-53	1.18	-	No procedure	Uveitis (C)
24	7-52	1.16	-	Enucleation for tumor	No tumor (P)
11	11-51	1.10	-	Reattached	Fluid detachment (C)
13	1-52	1.09	-	No procedure	Uveitis (C)
31	9-52	1.08	-	Reattached	Fluid detachment (C)
32	10-52	1.08	-	Reattached	Fluid detachment (C)
19	6-52	1.05	-	No procedure	Uveitis (C)
25	7-52	1.05	-	Reattached	Fluid detachment (C)
10	10-51	1.03	-	No procedure	Old healed inflam. lea (C)
41	4-53	1.02	-	No procedure	Benign Melanoma of Iris (C)
8	9-51	1.02	-	Enucl.-sec. glaucoma	Uveitis (chronic) (P)
20	6-52	1.02	-	Reattached	Fluid detachment (C)
1	12-50	0.98	1.00	Enucleation for tumor	Fluid detachment (P)
30	9-52	0.95	-	Retina completely det.	Fluid detachment (C)

Measured, but not included in this table are: 1 probable anterior tumor, refused enucleation, 8 cases with inconclusive results, to be observed and subjected to further measurements.

*By "proven" diagnoses is meant: 1. Definite pathological findings. 2. Successful reattachment. 3. Classical clinical findings.

Fig. 4 (Thomas, et al.) Summary of cases that received P^{32}.

Clinical results

Forty-four patients, many of whom had retinal separation and suspected tumors, have been subjected to study by this method. The data, including pathologic and clinical findings, on 11 cases of proved anterior tumors, three of proved posterior tumors, two of retinoblastoma, and 18 instances in which the lesions were not neoplastic are presented in Figure 4.

Ten of the 44 cases have not been tabulated because no definite clinical or histologic diagnosis has yet been made. In five of these 10 cases, the clinical changes and the abnormal P^{32} uptake ratios (in two instances, 2.4 and 1.6, respectively) indicate the probable presence of tumors. Two of these patients have so far refused enucleation, and the other three present posterior detachments on which preliminary measurements of radioactive phosphorus uptake have not been conclusive.

In the cases recorded, the ratio of selected uptake of tumor tissue to normal tissue averaged 1.77 for tumors in the anterior segment. The average ratio for non-neoplastic lesions of the anterior segment was 1.07. The average ratio for the neoplasms situated in the posterior segment averaged 1.26. It should be noted that in most of the cases here presented, the comparative counts were made only at intervals of 15, 30, and 60 minutes after injection, and were not studied in regard to the uptake immediately after introduction of the radioactive phosphorus.

In Cases 1 and 24, shown in Figure 4, the clinical evidence pointed strongly toward a diagnosis of tumor, so despite the fact that P^{32} uptake fell within the nontumor range, enucleation was performed. In both instances, histologic examination showed that no tumor existed.

Case 27 was one in which the clinical diagnosis appeared to be definitely that of a non-neoplastic fluid separation and reattachment was attempted. After six weeks, this proved unsuccessful and a P^{32} uptake study was then carried out. The test showed an increased ratio of 1.74. Because of the failure of the attempted reattachment and the finding of increased P^{32} uptake over the suspected area, the eye was enucleated and a melanoma was found.

In Case 15, a relatively high ratio was obtained during the first hour of counting. The average ratio of 1.3 included many counts over a considerable period of two months on repeated readings following three separate injections of the radioactive phosphorus. The output curve showed a high reading during the first hour and thereafter a drop to nearly 1.0. We believe this was due to the vascularity of the inflammatory process, which this lesion proved to be. In this instance, the true picture of the P^{32} output was obtained after most of the phosphorus had left the blood stream.

This series of cases includes two instances of retinoblastoma. In Case 37, it was possible to obtain counts only once before operation, and these were made during the first half hour after the injection of the P^{32}. Uniformly high counts over the tumor area and also over other portions of the eye possibly were an indication of an overshadowing by the vascular tissue of all the other tissue reactions. A more accurate reading might possibly have been obtained after three days, when most of the P^{32} would have been dispersed from the vascular system. The in vitro counts taken following enucleation four days after injection of the phosphorus showed a high ratio of 5.4 In Case 43, also of retinoblastoma, the readings were also taken early and here again only a fair differential between the counts over the pathologic and normal tissue was obtained, with a value of 1.15.

Discussion

There are many cases in which a trained ophthalmologist can make a satisfactory diagnosis on the basis of the appearance of a retinal separation. Nevertheless, there are numerous instances in which an elevation of the retina presents a difficult diagnostic problem.

When the detachment is fluid in nature and a retinal tear can be identified, the clinical evidence is strong that this lesion is due to a collection of subretinal fluid and that there is no neoplasm present.

In questionable cases when the retinal tear is not found and when the appearance of the separation is not characteristic, the test here described for measurement of P^{32} uptake appears to be an important aid in the diagnosis. Although all patients presenting retinal lesions may be studied by this means, there is some question as to whether all should be subjected to this test, or whether a system of screening should be followed.

The success of this test is greatly dependent upon the determination of the exact location of the separation and the site of the suspected tumor. Ophthalmoscopic examination must be carried out following the administra-

tion of a mydriatic, to permit the most careful localization.

It is necessary that the counter be in as close proximity to the tumor as possible, for, although the maximal range of the P^{32} beta particles is about eight mm., in tissue, the average range is only two mm. From the practical standpoint, a distance of five mm. or more between the tumor and the counter is unsatisfactory. Therefore, when difficulty is encountered in proper placement of the counter in close apposition to the tumor, the significance of the result, particularly if it be a negative one, is doubtful. The difficulty in studying tumors in the extreme posterior quadrants has already been mentioned.

Although we have examined 44 cases, the results must still be used with considerable caution. It is certainly not intended to propose that the selective uptake of P^{32} in tumors be utilized as a substitute for a thorough and careful clinical examination of a patient with a suspected intraocular tumor. Nevertheless, from the data we have obtained, it does appear to be a valuable adjunct. The work is to be continued with special emphasis on the development of a counter which will permit satisfactory measurements over the posterior segments of the globe. Complete understanding of the practical usefulness of the test must await much more extensive application.

Summary

Forty-four cases of various intraocular lesions have been studied. Measurement of the differential uptake of P^{32} in tumors appears to indicate a clear separation of neoplastic lesions from non-neoplastic disease. The test is most effective when applied to lesions in the anterior segment of the eye.

In efforts to improve the validity of this test for the diagnosis of neoplasms situated in the posterior portion of the globe, an improved type of counter with a 45-degree angle-window has been found to be more satisfactory than the small end-window counter originally used.

Retinoblastoma has presented several unique problems. The differential between normal and pathologic tissue which is seen from two to three days after injection of P^{32} is not apparent in readings made immediately after injection of the radioactive material. Perhaps the type or mode of extension of the tumor growth or the vascularity over the tumor and the tissue surrounding it affects the rate of uptake of radioactive phosphorus, or masks its presence.

The determination of the P^{32} uptake is offered as an adjunct to careful clinical evaluation of suspected intraocular tumors, rather than as a conclusive diagnostic test.

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Discussion

Dr. Irving H. Leopold (Philadelphia): It should be noted that Dr. Thomas and his associates are pioneers in the application of radioactive phosphorus in the clinical diagnosis of intraocular neoplasms. Experimental work in intraocular neoplasms had been reported previously by Dunphy and his associates, but the first clinical trials that were reported in the ophthalmologic literature were those of Dr. Thomas and his co-workers.

Dr. Thomas has concluded that anterior-segment lesions can be detected with a fairly high degree of accuracy by the presently available probes and Geiger counter, but that posterior-segment lesions are rather difficult to detect because of the inaccessibility of the lesion. These conclusions are almost identical with those reported by Dunphy and his associates at the American Ophthalmological Society, and reported this morning by Dr. Eisenberg at the A.M.A. Section meeting.

Dr. Thomas has called attention to the importance of the time at which readings are made after the injection of P^{32}. One case was that of a youngster with retinoblastoma, in whom the initial readings were low, following the injection of P^{32}, but after a day or two, the readings became detectably different between the site of the lesion and the normal tissue.

Another was an instance of inflammatory tissue in which the readings were high after the injection of P^{32}; and as the time elapsed, the difference between the inflammatory lesion site and the normal tissue dropped. It is possible, therefore, that by altering the time at which readings are taken after intravenous injections of P^{32}, one may be able to clarify further the differential diagnosis of intraocular and inflammatory lesions.

There are many unexplained elements in this test which still await solution. Dr. Thomas mentioned a few of these, namely, that one can still not be certain as to the method by which P^{32} is localized in the intraocular lesion. Is it, as Dr. Dunphy suggested, simply the mass of the lesion? The lesion at that site is a tumor, and takes up more space, and therefore holds more P^{32} than the adjacent thinner, smaller, normal tissue. Or, is it due to increased cellular metabolism of the neoplasm? Or, is it due to increased blood supply?

What effect will P^{32} have on eyes which have recently been operated on for retinal detachment? For example, if one were to see a retinal detachment operated unsuccessfully, and then begin to wonder whether there might be a malignant melanoma, and decide to run a P^{32} test or study, would it be possible that these diathermy lesions might cause a concentration of P^{32} which would give a false positive?

Also, what are the influences of conjunctivitis and local superficial inflammations? All these things have to be worked out; but with the many interested research groups throughout the country, the answers do not seem too far away.

Dr. Frederick Blodi (Iowa City, Iowa): I can only confirm the reports that it is fairly valuable and easy to pick up differences in tumors or cellular masses in the anterior segment, but it is extremely difficult in our hands and almost impossible to do in cellular masses in the posterior part of the globe.

There is one point which I would like to make, however, and one more difficulty and fallacy of this test. In the case of the little girl who came to the University Hospital with a diagnosis of retinoblastoma, we assumed we were dealing with a simple detachment. After making a radioactive phosphorus test, we found the uptake was the same in both eyes. However, on the assumption that we could not pick up radioactive phosphorus from a deeper tumor, we tried after some time to measure the secondary gamma radiation emitted assumedly by the radioactive phosphorus in this tumor, and to our surprise we found that the diseased eye emitted more gamma rays than the normal eye.

This made us very suspicious of a tumor. When we enucleated the eye and opened it, we found a simple serous detachment.

Dr. Jack S. Krohmer: I thought it worth while to mention that we have found that inflammatory lesions give a high ratio. However, since this is predominantly due, we think, to increased vascularity, it stands to reason that this ratio should decrease in a fairly short period of time; and we have found that, in general, this is the case. Even though we may get a high ratio initially, it falls and has gone down to a fairly low value by the end of an hour.

In most of the tumor studies we have stated ratios of counts and actually we have taken the peak-to-peak ratio. It turns out that the uptake in any tissue goes up to a peak and then starts down. However, we did not consider the peak-to-peak unless it stayed up to a fairly high value for a period of at least an hour; and this, I think, accounts for some differentiation and is a means of differentiation between malignant lesions and inflammatory lesions.

I don't know whether that has been found by others who have investigated it, but I think it logically follows. However, this is not always a good way to decide things.

There was a statement made about gamma-ray counting. In some instances we have counted over the eyes, using a gamma emitter. In this case you are practically limited to counting the affected eye as opposed to the other eye. I think you are probably all aware of the fact that the range of gamma radiation is quite large, and that one can get a fairly sizable count from portions other than the eye.

That is why we have never felt that it is as good as a beta emitter, although in some cases we have been able to detect tumors. We feel that a beta-ray count over the tumor, as opposed to a count over another section of the same eye, is the best indicator.

INVESTIGATION OF CORTICOSTEROIDS IN THE AQUEOUS HUMOR OF NORMAL ANIMAL EYES*

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The increasing use of cortisone (compound E) and hydrocortisone (compound F) in the treatment of inflammatory conditions of the anterior segment of the eye prompted this investigation. We deemed it of primary interest to establish whether corticosteroids are present in the normal primary aqueous humor and at what concentration. Perhaps these studies will shed some light upon the relationship of corticosteroids in the aqueous humor with ocular disturbances and diseases, and with general physiologic factors that may predispose to the development of such conditions.

MATERIALS AND EQUIPMENT

All organic solvents were redistilled prior to use. Chloroform and methanol were Baker's analyzed, reagent grade; phenylhydrazine hydrochloride and tetramethylammonium hydroxide (10-percent aqueous solution) were Eastman Kodak products; blue tetrazolium, Dajac laboratories; crystalline corticosteroids;† β-glucuronidase, Viobin. Beckman Spectrophotometer, Model DU; Evelyn colorimeter; Cambridge pH meter, model L.

EXPERIMENTAL

PROCEDURE

Normal eyes of adult rabbits were used. Anesthetization was effected by the instillation of 0.5-percent pontocaine hydrochloride. The eyes were then gently and thoroughly flushed with physiologic saline solution. Care was taken to avoid excitation of the animal.

The aqueous humor was removed by an oblique puncture of the corneal-scleral limbus by means of a No. 26-gauge needle fitted to a tuberculin syringe and immediately emptied into redistilled dry chloroform in a separatory funnel. The aqueous humor was extracted with three to five portions of chloroform‡ and the latter was removed by means of a vacuum pump at a bath temperature of 45°C.

The resulting residue was dissolved in an appropriate solvent and analyzed. Unless otherwise indicated a sample equivalent to 0.2 to 0.5 ml. of original aqueous humor was used for analysis.

RESULTS

Ultraviolet absorption spectrum. The presence of the α, β-unsaturated ketonic grouping at carbon atoms 3 and 4 (fig. 1) is readily identified by the appearance of an absorption peak at 240 mμ. Approximately 2.0 μg. cortisone per ml. of absolute alcohol can be detected in this way. The ultraviolet absorption curve of pooled aqueous humor, however, in quantities up to 1.3 ml. per ml. of absolute alcohol showed no absorption peak in this range.

The absorption curve possessed a maximum at 305 to 310 mμ, under the conditions of the experiment. That this peak was due to the pontocaine used in the anesthetization was demonstrated by removing the aqueous humor under general anesthesia by the intra-

* From the Department of Research, Wills Eye Hospital. This investigation was supported by a research grant from The National Institute of Neurological Diseases and Blindness of the National Institutes of Health, United States Public Health Service.

† We gratefully acknowledge the samples of pure crystalline corticosteroids generously donated by the following: Merck and Co., Inc.; Ciba Pharmaceutical Products, Inc.; Parke, Davis and Co.; The Upjohn Company.

‡ In order to avoid the formation of difficult emulsions it was necessary to use at least six parts by volume of chloroform to one of aqueous humor.

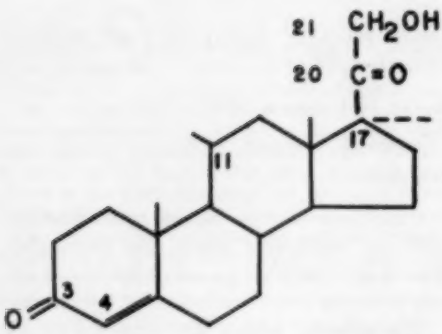


Fig. 1 (Green, Weimar, and Leopold). Skeletal structure of the corticosteroids. The numbers indicate the positions of the constituent functional groups. The indicated structures of the six "active" corticosteroids are:

1. Cortisone (E) 17-OH, 11-C=O
2. Hydrocortisone (F) 17-OH, 11-OH
3. 17-Hydroxydesoxycorticosterone (S) 17-OH
4. 11-Dehydrocorticosterone (A) 11-C=O
5. Corticosterone (B) 11-OH
6. Desoxycorticosterone

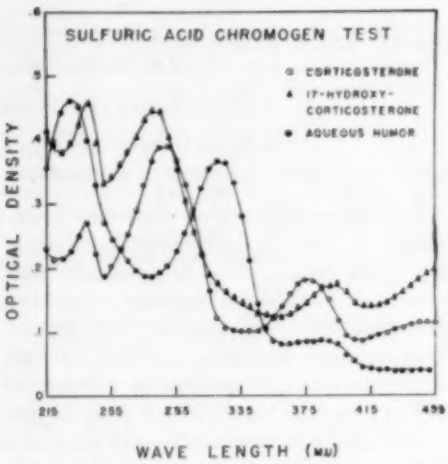


Fig. 2 (Green, Weimar, and Leopold). Absorption curve of the sulfuric acid chromogen.

venous administration of sodium nembutal. Under these conditions the peak at 305 to 310 mμ disappeared.

In addition, the ultraviolet spectrum of a solution of pontocaine showed this characteristic absorption peak. The presence of traces of residual chloroform also contributed an absorption peak at 215 mμ.

Absorption spectrum of sulfuric acid chromogen. The method employed was essentially that of Zaffaroni[1] with the Goldzieher, Bodenchuk, and Nolan[2] modification of heating at 80°C. for 10 minutes.

Because of the small quantities of materials being measured the volume of H_2SO_4 used was reduced to 1.0 ml. Figure 2 compares the absorption curves of the sulfuric acid chromogens produced by corticosterone and 17-hydroxycorticosterone with that of aqueous humor. It can be seen that all three curves have maxima at 230 to 240 mμ and at 375 to 395 mμ. In addition corticosterone and 17-hydroxycorticosterone have maxima at 280 to 285 mμ and at 455 to 475 mμ, while aqueous humor possesses a maximum at 320 mμ.

Porter-Silber test.[3] This test is specific for the detection of the 17, 21-dihydroxy-20-keto side chain of corticosteroids. Pooled aqueous humor in quantities up to 1.7 ml. did not give a positive reaction with this test.

Blue Tetrazolium test. A modification of the method of Mader and Buck[4] was employed: the evaporated residue of the chloroform extract of aqueous humor was dissolved in 1.0 ml. of 95-percent ethanol. To this was added 0.2 ml. of 0.11 N tetramethylammonium hydroxide* and 0.1 ml. of 0.5-percent alcoholic solution of Blue Tetrazolium. The color was developed at room temperature for 60 minutes.†

* This increased quantity of tetramethylammonium hydroxide gave a more intense and faster color development.

† A 20 to 30 percent increase in intensity of color can be obtained by heating the reaction mixture of the aqueous humor at 50°C. for 15 minutes, after the 60 minutes at room temperature. This procedure, however, has no effect upon the color development of desoxycorticosterone used as a standard. On the assumption that this heat-responsive increase was due to the presence of less readily hydrolyzable steroid esters, the samples of extracted material were submitted to varying conditions of acid and alkaline hydrolysis prior to color development at room temperature. In all cases there was either no effect or, more often, destruction of Blue Tetrazolium-positive material. This observation remains unexplained.

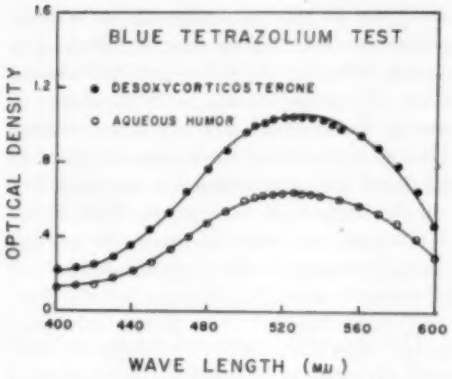


Fig. 3 (Green, Weimar, and Leopold). Absorption curve of the Blue Tetrazolium chromogen.

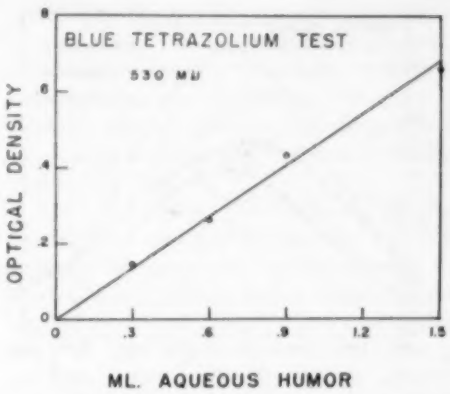


Fig. 4 (Green, Weimar, and Leopold). Variation of the optical density of the Blue Tetrazolium chromogen with the concentration of aqueous humor.

Since the intensity of the color produced is directly proportional to the concentration of the α-ketol side chain and practically independent of the rest of the molecule, this test was readily adapted to quantitative evaluation with the aqueous humor. Figure 3 gives the absorption curve of a representative corticosteroid, desoxycorticosterone, and a typical curve produced by aqueous humor after reaction with Blue Tetrazolium.

The shapes of the curves are very similar with a maximum at 530 mμ. Moreover, the intensity of the color is directly proportional to the quantity of aqueous humor, as shown in Figure 4. By measuring the absorption at 530 mμ we have repeatedly obtained the value of between 2.0 and 4.0 μg. of corticosteroid, in terms of desoxycorticosterone, per ml. of the original primary aqueous humor. This method has permitted us to determine as little as 0.5 μg. of steroid.

Effect of conditions of evaporation upon recovery yields

During the initial phase of this investigation, the chloroform extract was evaporated by means of a water aspirator. Under these conditions inconsistent and variable recovery yields of Blue Tetrazolium-positive material were obtained. Subsequently, in order to facilitate the handling of large pools of aqueous humor, we shifted to the use of a vacuum pump. With the latter, as mentioned previously, reproducible values of 2.0 to 4.0 μg. of corticosteroids per ml. of original aqueous humor were obtained.

Further experimentation with the use of a water aspirator, a stream of nitrogen gas, and just gentle heating to remove the chloroform resulted in markedly higher recovery yields of Blue Tetrazolium-positive material. Indeed, these high values could be lowered to the 2.0 to 4.0 μg. level by continuing the evaporation with a vacuum pump.

Control recovery experiments were run with cortisone and desoxycorticosterone dissolved in the chloroform extracts of the aqueous humor. In all cases the pure corticosteroids were quantitatively recovered as measured by the Blue Tetrazolium test. In this regard, the results with the Porter-Silber tests are of interest.

As previously mentioned, the material recovered by evaporation of the chloroform with a vacuum pump did not give a positive response with this test. However, the material recovered under the milder conditions of evaporation gave a marked response to the Porter-Silber test. This is shown in Figure 5.

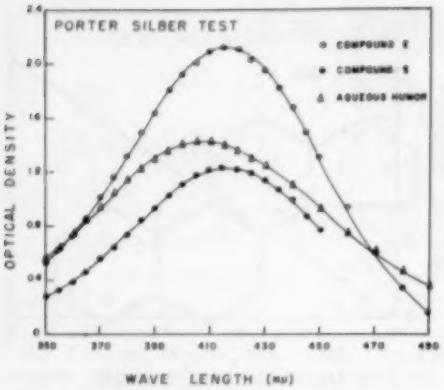


Fig. 5 (Green, Weimar, and Leopold). Absorption curve of the Porter-Silber chromogen. Concentration of cortisone and 17-hydroxydesoxycorticosterone, 8.33 μg/ml. of reaction mixture.

The absorption curves for the reaction products of equal concentrations of cortisone and 17-hydroxydesoxycorticosterone with this test are also given for purposes of comparison, and to show that the extent of the response is dependent upon the particular steroid. Consequently, the Porter-Silber test cannot be used for quantitative determination of mixtures of unknown corticosteroids.

Some preliminary experiments have been done on repeated paracenteses of the anterior chamber of rabbit eyes at 20-minute intervals. The results indicate an immediate increase in material, recovered from the chloroform extract by means of a vacuum pump, that gave a positive response to the Blue Tetrazolium test. It is further significant that this heightened level of activity was maintained during the next three taps and then tended to fall.* These experiments are continuing.

Action of β-glucuronidase

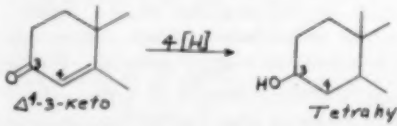
In order to determine whether any glucuronides of the corticosteroids were present, the aqueous humor was incubated in an acetate buffer at pH 4.5 with 500 Fishman[6] units of β-glucuronidase at 38°C. After 15 hours, the reaction mixture was extracted with chloroform and the residue obtained by means of a vacuum pump was analyzed. The results showed no increase in Blue Tetrazolium-positive material over the control sample untreated with β-glucuronidase.

Discussion

The absorption spectrum of the sulfuric acid chromogen is usually employed as a possible criterion for the presence of corticosteroids. The absorption peaks of the spectrum are believed to be characteristic of the functional groups, although each steroid gives its own peculiar spectrum.

An examination of absorption curves of the sulfuric acid chromogens produced by the known corticosteroids strongly suggests that the peak at 230 to 240 mμ is associated with the 11-OH group; the peak at 280 to 285 mμ is generally accepted as revealing the presence of the α-keto-β-unsaturated grouping at positions 3 and 4, which is absent in the spectrum of the aqueous humor (fig. 2). This is in agreement with the fact that such a group could not be detected in the ultraviolet absorption spectrum of the aqueous humor.

The appearance of the 320 mμ peak in the sulfuric acid chromogen absorption curve of aqueous humor and the absence of the 280 mμ peak suggested the possible reduction of the Δ^4-3-keto group. Indeed the sulfuric acid chromogen absorption curves of tetrahydro E and tetrahydro F, reduction products of E and F, respectively, in the 3, 4 positions, are reported to have a peak at



330 mμ; also the peak formerly present at 280 to 285 mμ in the absorption curves of the

* Leopold and Maylath[5] working with the whole aqueous humor showed an increase in Porter-Silber positive material with repeated paracenteses at one-hour intervals. No attempt was made by these investigators to remove the glucose or other materials that react with the Porter-Silber reagents.

original unreduced corticosteroids has disappeared. From these data it is reasonable to assume that the peak at 320 mμ in the absorption curve of the aqueous humor is associated with the reduction of the Δ^4-3-keto grouping. Attempts to separate and identify the corticosteroids in the aqueous humor by paper chromatography are now in progress.

To date about 28 crystalline compounds have been isolated and characterized from beef adrenal extracts and there remains an unknown number of unidentified physiologically active compounds. At least half of the known corticosteroids possess the α-ketol side chain. This is the only functional group possessed by all of the known active corticosteroids. This unique property makes the quantitative evaluation of the α-ketol group of great analytical value.

In this regard the Blue Tetrazolium test, specific for the detection of the α-ketol group, is an excellent analytic tool in the quantitative assay of pure corticosteroids. Moreover, since the magnitude of the color development in this test is directly proportional to the concentration of the functional group, and practically independent of the rest of the steroid molecule, this test is most useful. By this means and with the use of a vacuum pump to remove the chloroform, we have established the level of probable corticosteroids in 1.0 ml. of normal primary aqueous humor to be 2.0 to 4.0 μg.

The fact that markedly higher values have been obtained under conditions of evaporation less exhaustive than with the use of a vacuum pump suggested the presence of different molecular species in the extract. This has been partly confirmed by the finding that the more volatile material possessed the properties of a 17, 21-dihydroxy-20-keto side chain (Porter-Silber test), while the material recovered with the use of a vacuum pump did not possess this functional group.

It is interesting to speculate that the more volatile material may contain metabolic products of the corticosteroids. Our observations seem to be the first to suggest a dependence of the recovery of corticosteroids or corticosteroid-like material from body fluids upon the conditions of evaporation of the extracts thereof. Work is now in progress designed to separate and characterize the more readily vaporizable fraction.

The preliminary observations with repeated paracenteses of the anterior chamber indicate that accompanying the entrance of the secondary aqueous humor there is an increased amount of Blue Tetrazolium-positive material. This heightened level is apparently unaffected during the 20 minutes between taps. These experiments are continuing and it will be of interest to see the relationship between the time-interval of paracenteses and the level of activity in the aqueous humor; and, further, to determine the time dependency for biologic transformation of the corticosteroids in the aqueous humor.

It has already been suggested by Leopold and Maylath[5] that the "beneficial effects of plasmoid aqueous in the therapy of various inflammatory conditions of the anterior segment of the eyes, such as uveitis and keratitis, may in part be due to this increased quantity of corticoid materials in the plasmoid aqueous." Experiments designed to test this hypothesis are now in progress.

Summary

Evidence has been presented suggesting the presence of corticosteroids in the aqueous humor of normal rabbit eyes. The concentration of recoverable steroidlike material depends upon the conditions of evaporation of the chloroform extract, the minimum value being 2-4 μg/ml. of original aqueous humor. There is no reason to believe that the structural composition of the more volatile fraction may be different from the nonvolatile fraction. The data presented indicate the absence of the Δ^4-3-keto grouping, and suggest that this structure may be present in its reduced form. There is also presumptive evidence for the presence of a

11-OH group in the nonvolatile fraction and the appearance of a 17-OH group in the more volatile fraction. Repeated paracenteses of the anterior chamber lead to an immediate increase in Blue Tetrazolium-positive material.

1601 Spring Garden Street (30).

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Discussion

Dr. John Harris (Portland, Oregon): I can't refrain from commending the authors on such a tremendous piece of work. As Dr. Green pointed out, it is a terrific task to separate these steroids, and it is a task which sometimes is a thankless one. Yet if we are to understand completely the effect of the corticosteroids on disease, and if we are ever to use them as the tool that Thorne has suggested, namely, to determine the etiology of pathologic processes that are altered by the hormones, such studies as Dr. Green, Dr. Weimer, and Dr. Leopold have reported are essential.

I am particularly interested in one aspect of this problem. Mencken was able to demonstrate a measurable concentration of isotopically labeled compound E at a chemically inflamed area of the pleura. In other words, he felt that this steroid was possibly attracted to an inflammatory site.

I am wondering whether the results which Dr. Green and his colleagues obtained after paracentesis can be interpreted in a similar manner.

Dr. Harry Green (Philadelphia): Our results refer to normal primary aqueous humor. The significance of the maintenance of the level of corticosteroids after paracenteses at 20 minute intervals can be speculated upon, but I think, perhaps, the discusser was referring to inflammatory conditions of the anterior segment. Under these conditions, presumably, the response of the organism may alter the level of activity of the corticosteroids within 20 minutes or less.

I would like to take this opportunity to add that at the present time we are attempting to separate and characterize these corticosteroids in the aqueous humor by paper chromatography, and we also are attempting to separate quantitatively and further characterize the more volatile fraction as presented in this work.

STUDIES ON THE NATURE OF THE EXOPHTHALMOS-PRODUCING PRINCIPLE IN PITUITARY EXTRACTS*

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New York

Exophthalmos is so associated with thyroid disease that the two are linked in the name "exophthalmic goiter," even though in a large proportion of the cases no exophthalmos is evident. During the past 20 years the closeness of this association has decreased in the minds of some investigators, as it has become evident that exophthalmos is not directly caused by the thyroid hormone. However, in the experiments of Shockaert[1] and of Loeb,[2] when exophthalmos was produced by the injection of hypophyseal extracts in ducks and guinea pigs, it was generally assumed that it was in some way caused by the thyrotropic hormone which the extracts contained.

The demonstration[3, 4] that it was readily produced by the injection of pituitary extracts, in the absence of thyroid gland, did not lessen the generally accepted concept that the active principle causing the exophthalmos was thyrotropic hormone, even though all preparations used were rich in other hypophyseal hormones in addition to the thyrotropic. No proof was advanced demonstrating either that the exophthalmos was or was not caused by the thyrotropic hormone, and the expression "thyrotropic exophthalmos" became common in the literature without adequate justification.

Several studies have been reported in which the role of the thyroid stimulating hormone in the production of exophthalmos has been investigated. Albert[5] concluded that the thyrotropic principle was responsible for exophthalmos produced in fish. It has been reported[6, 7] that ACTH at least was not the causative agent.

Dobyns[8] questioned the role of thyrotropic hormone in exophthalmos, because he found that a purified thyrotropic preparation did not produce it. Jeffries[9] came to a similar conclusion based upon an experiment in which iodination of a pituitary extract was reported to reduce its thyrotropic more than its exophthalmos producing potency. In contrast to this, however, Dobyns and Rawson,[10] in a most ingenious experiment, found that thyroid tissue, incubated in a pituitary extract, inactivated the thyrotropic principle without appreciably affecting others, such as the gonadotropic hormone. Extracts treated in this way did not cause exophthalmos. If one assumes that thyroid tissue acts very specifically, removing only the thyrotropic hormone from solution, the data support the contention that exophthalmos is caused by the thyroid-stimulating material.

Another possibility, which has received rather recent attention, is that exophthalmos may result from the interaction of more than one hormone. Aterman[11] suggested that it occurs as the result of simultaneous action of the thyrotropic and adrenocorticotropic hormones (ACTH). The synergistic action of hypophyseal hormones is not without precedent, for example, the effect of thyrotropic and growth hormones on body growth, or of the luteinizing and follicle-stimulating hormone on ovarian growth and estrogen production.

Exophthalmos in guinea pigs results from the increase in space occupied by several orbital structures, orbital fat, the Harderian gland, and the extraocular muscles. Orbital fat increase is mainly due to imbibition of water (fig. 3). Growth of the Harderian gland appears to be a normal hyperplasia and hypertrophy associated with an increase in secretion.[12] Muscle hypertrophy is due in part to the same process as causes that in fat

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but, in addition, normal work hypertrophy also apparently occurs.[12]

Any analysis of endocrine factors involved in production of exophthalmos must take account of the diverse effects of the extracts on the orbital structures, which result in protrusion of the globe. This means that the effect of a given preparation on the weight of all tissues, and their histologic modification, must be determined in addition to a diagnosis of exophthalmos based on the subjective impression by an observer, or on measurements made of the ocular protrusion in the intact animal.

Experimental

A series of experiments was undertaken to clarify the relation of the known anterior pituitary factors to experimentally produced exophthalmos in guinea pigs. These may be divided into four parts:

I. A quantitative study was carried out to determine whether or not the degree of exophthalmos produced, and the modification of orbital content, was proportional to the amount of crude pituitary extract injected.

II. The effect of injection of single relatively purified anterior pituitary principles on exophthalmos and orbital modification was determined.

III. The action of a combination of relatively purified anterior pituitary hormones on the protrusion of the globe and orbital contents was studied. This mixture was rich in the well-known pituitary principles excepting thyrotropic hormone.

IV. Crude extracts of the hypophysis of several species—beef, sheep, pig, and horse were compared in their ability to produce exophthalmos and in their content of the thyrotropic and other anterior pituitary hormones.

I. Quantitative study

For these experiments a stock extract of acetone dried beef anterior pituitary glands was prepared. The material, made according to a method reported earlier,[6, 14] was well tolerated even when large doses were injected daily. It contained all of the well-known endocrine principles of the anterior hypophysis: thyrotropic hormone (TSH), adrenocorticotropin (ACTH), growth, prolactin (luteotropin), and gonadotropins (luteinizing hormone [LH], and follicle-stimulating hormone [FSH]).

The "assay" of exophthalmos-producing potency was carried out, after some tentative experiments, in a manner which was rigidly adhered to in all experiments. Female guinea pigs of mixed strains were obtained just after weaning, at 200 to 220 gm. body weight, and thyroidectomized when they had attained a weight of 270 to 280 gm. They were maintained on a diet of Rockland Farm pellets plus greens, chiefly lettuce. Water was always available.

Ten days post-thyroidectomy the guinea pigs were injected subcutaneously with 0.5 to 1.0 cc. of the test substance, in saline, daily for 12 days and autopsied on the 13th day.

At autopsy, in addition to the subjective estimation of exophthalmos, the protrusion of the eyes was measured post mortem as described earlier.[13] The distance from the limbus to the superior orbital rim at the supraorbital notch was determined with Vernier calipers, and recorded as a measure of exophthalmos (graph 1). Orbital fat, extraocular muscles, Harderian and lacrimal glands, and globe were weighed, and the fat, muscle, and Harderian glands were fixed for histologic study.

The interorbital distance was determined by caliper measurement and regarded as a criterion of the size of the guinea pigs used. The weights of the adrenal glands, ovaries, and uteri were also determined, and served to indicate the quantity of ACTH and gonadotropins which the extracts contained.

In all cases a careful search for thyroid remnants was made at autopsy. Cases of incomplete thyroidectomy were discarded.

Since animals of the same age and size were used in these experiments, very little

variation in body and eyeball weight or interorbital distance was found at autopsy. Consequently the weights of various structures shown in graphs 1 to 6 may be freely compared. The PMS series is an exception, for these animals were older and larger than in the other groups.

The stock exophthalmos-producing extract of beef pituitary glands was carefully assayed for each of the well-known pituitary hormones. The thyrotropic hormone content was assayed on chicks according to the method introduced earlier.[14] Ten chicks were used at each dose level and a standard was included in each assay.

The amount of material necessary to produce a definite thyroid hypertrophy was regarded as the unit in these experiments, and was equivalent to 50 gamma of the U.S.P. standard.

The gonadotropic potency was assayed on mice, according to the method of Levin and Tyndale,[15] and in hypophysectomized rats.[16] Prolactin was tested by the crop gland technique in white Carneau pigeons following the method of Bates.[17] Growth hormone activity was determined by utilizing the increase in the tibial cartilage of the hypophysectomized rat, as suggested by Greenspan, et al.[18] ACTH was estimated by the adrenal hypertrophy produced in hypophysectomized rats and by the histologic redistribution of adrenal lipids.[19]

Both the ovarian and adrenal hypertrophy produced in the thyroidectomized guinea pigs in the tests made for exophthalmos were of the greatest value in estimating the activity of these two principles contained in the extracts. It is exceedingly difficult, if not impossible, to determine the quantity of some of these hormones when in mixture with others (for example, growth and ACTH). This problem is recognized, if not solved.

Twenty-one guinea pigs were reserved as uninjected controls. They were of the same age and weight, and thyroidectomized for the same length of time as were the experimental animals. In addition, they were collected throughout the entire period of this investigation so that they served all experimental groups as a basis of comparison.

Six animals were injected with 5.0 mg. of the stock beef extract daily. No case of definite exophthalmos was produced. Modification of the orbital content was slight.

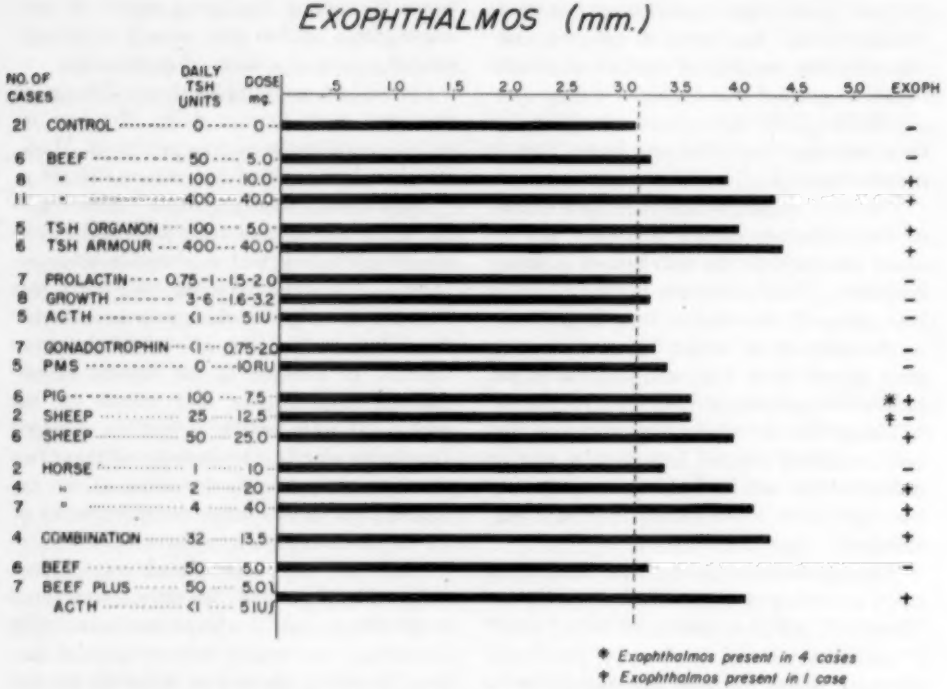
Eight other guinea pigs were injected with 10 mg. of the stock extract daily and a definite exophthalmos was produced in seven instances. A thyroid remnant (4.2 mg.) was found in the eighth, which may account for the failure in this case. In each of the seven animals, in addition to the definite exophthalmos, hypertrophy and edema of the orbital fat were noted, as well as enlarged Harderian glands. The increase of these two tissues was apparently the cause of the exophthalmos. In this series no hypertrophy of the extraocular muscles was noted.

Another group of 11 animals was injected daily with 40 mg. of the same extract and exophthalmos was produced in all cases. The degree of protrusion, hypertrophy of fat, and Harderian gland was definitely greater than in the series receiving a smaller quantity of pituitary extract. In addition to these changes, hypertrophy of the extraocular muscles also occurred.

The above results, together with those of subsequent experiments, are summarized in Graphs 1 to 6.

A second quantitative experiment was performed with another crude pituitary extract in which 15 guinea pigs were divided into control and injection groups. In this experiment, it was found that the degree of exophthalmos and orbital modification also could be correlated with the amount of pituitary extract given.

It is apparent that the degree of exophthalmos and changes in the orbital tissues may be directly related to the amount of exophthalmos-producing substance injected. Furthermore, of this particular beef pituitary extract, 10 mg. was approximately the minimal amount necessary to produce this effect. Biologic assay of this stock material showed that


EXOPHTHALMOS (mm.)
NO. OF CASES
DAILY TSH UNITS
DOSE mg.
.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5 5.0 EXOPH
21 CONTROL 0 0 -
6 BEEF 50 5.0 -
8 " 100 10.0 +
11 " 400 40.0 +
5 TSH ORGANON 100 5.0 +
6 TSH ARMOUR 400 40.0 +
7 PROLACTIN 0.75-1 1.5-2.0 -
8 GROWTH 3-6 1.6-3.2 -
5 ACTH <1 5 IU -
7 GONADOTROPHIN <1 0.75-2.0 -
5 PMS 0 10 RU -
6 PIG 100 7.5 * +
2 SHEEP 25 12.5 ‡ -
6 SHEEP 50 25.0 +
2 HORSE 1 10 -
4 " 2 20 +
7 " 4 40 +
4 COMBINATION 32 13.5 +
6 BEEF 50 5.0 -
7 BEEF PLUS ACTH 50 5.0 <1 5 IU +
* Exophthalmos present in 4 cases
‡ Exophthalmos present in 1 case

Graph 1 (Smelser and Ozanics). Studies on the exophthalmos-producing principle in pituitary extracts.

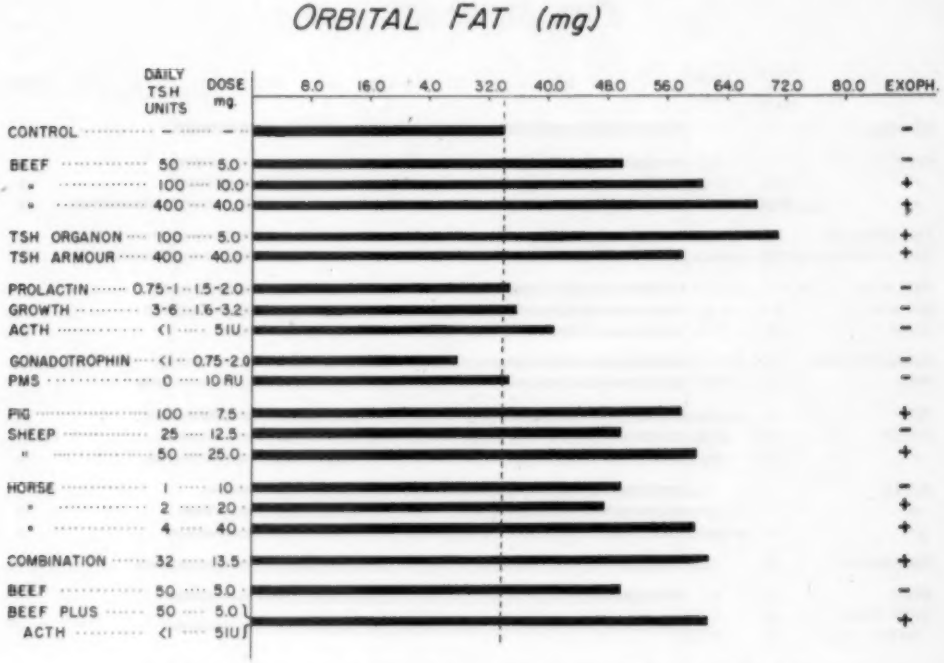
TABLE 1

RELATIVE HORMONAL ACTIVITY OF THE PREPARATIONS USED

(The quantity of each preparation is shown which evoked a response in the test object equivalent to that produced by a standard.)

	Hormone	Thyrotropic Hormone	Gonadotropin		ACTH	Growth	Prolactin
	Test object	Chick thyroid wt.	Immature mouse uterine wt.	Hypophysect. rat ovarian wt.	Hypophysect. rat adrenal wt.	Hypophysect. rat tibial cartilage width	Pigeon crop sack
	Standard of comparison	50γ USP thyrotropic substance	0.5 mg. Beef prep.	2.5 mg. Beef prep.	2.5 mg. Beef prep.	2.5 mg. Beef prep.	20.0 mg. Beef prep.
Pituitary Preparations	Beef	0.1 mg.	0.5 mg.	2.5 mg.	2.5 mg.	2.5 mg.	20.0 mg.
	Pig	0.075 mg.	0.2 mg.	2.5 mg.	2.5 mg.	> 2.5 mg.	—
	Sheep	0.5 mg.	0.1 mg.	10.0 mg.	10.0 mg.	10.0 mg.	—
	Horse	10.0 mg.	<0.025 mg.*	*	10.0 mg.	10.0 mg.	—
	TSH (Organon)	0.05 mg.	—	2.5 mg.	2.5 mg.	> 2.5 mg.	—
	TSH (Armour)	0.1 mg.	0.5 mg.	10.0 mg.	10.0 mg.	>10.0 mg.	20.0 mg.
	LH+FSH	> 2.0 mg.	0.00625 mg.	*	>2.0 mg.	2.0 mg.	2.0 mg.
	FSH	2.0 mg.	0.25 mg.	—	—	—	—
	ACTH	>10.0 IU	—	5.0 IU	5.0 IU	> 5.0 IU	—
	Growth	0.5 mg.	0.5 mg.	> 0.05 mg.	0.05 mg.	0.05 mg.	—
	Prolactin	2.0 mg.	2.0 mg.	>10.0 mg.	10.0 mg.	2.5 mg.	0.5 mg.

* These preparations were not tested at sufficiently low dose levels. The response evoked was far greater than that produced by the standard preparation.


ORBITAL FAT (mg)
DAILY TSH UNITS
DOSE mg.
8.0 16.0 4.0 32.0 40.0 48.0 56.0 64.0 72.0 80.0 EXOPH.
CONTROL – – –
BEEF 50 5.0 –
" 100 10.0 +
" 400 40.0 +
TSH ORGANON 100 5.0 +
TSH ARMOUR 400 40.0 +
PROLACTIN 0.75-1 1.5-2.0 –
GROWTH 3-6 1.6-3.2 –
ACTH <1 5IU –
GONADOTROPHIN <1 0.75-2.0 –
PMS 0 10 RU –
PIG 100 7.5 +
SHEEP 25 12.5 –
" 50 25.0 +
HORSE 1 10 –
" 2 20 +
" 4 40 +
COMBINATION 32 13.5 +
BEEF 50 5.0 –
BEEF PLUS 50 5.0 +
ACTH <1 5IU

Graph 2 (Smelser and Ozanics). Studies on the exophthalmos-producing principle in pituitary extracts.

it contained 10 thyrotropic units per mg. (one unit = 50 gamma U.S.P. thyrotropic preparation).

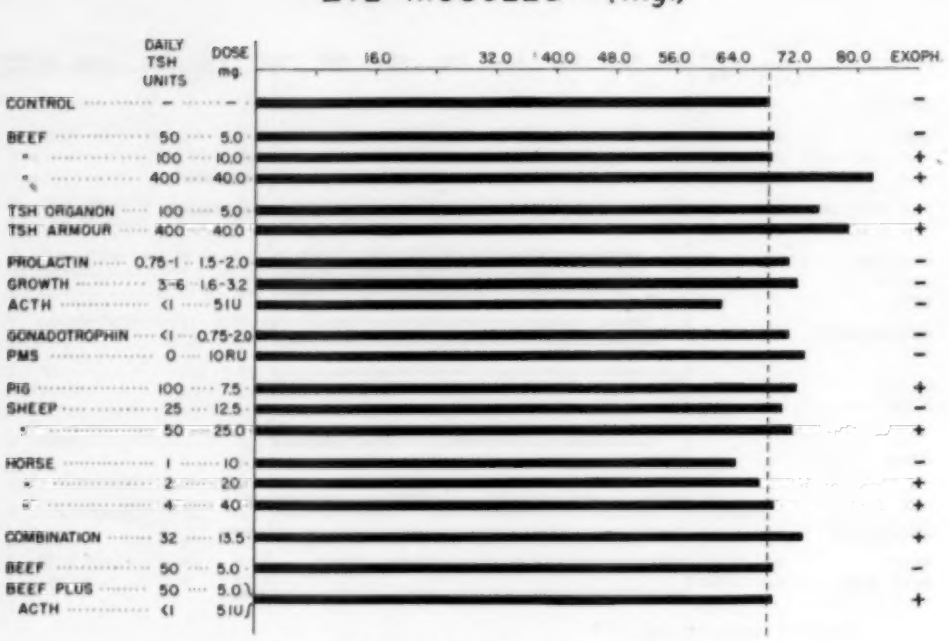
Tests for gonadotropic, growth, and ACTH showed that considerable quantities of these hormones were also present. A rather small amount of prolactin was found since 20 mg. of this preparation was required to produce a definite crop gland response. Since exophthalmos was produced by this heterogeneous material, it was obviously necessary to determine whether any of the above hormones were separately responsible for it.

II. Effect of relatively purified anterior pituitary principles in exophthalmos

Preparations of growth hormone, ACTH, follicle-stimulating hormone, thyrotropic hormone, gonadotropin, and prolactin, each partially purified and containing predominantly a single principle, were obtained. Each of these preparations was more or less contaminated with other hormones and unknown substances, which might or might not have had physiologic activity. The results of the assay of these preparations and of the stock beef pituitary extract are given in the table.

Each of the hormone preparations was injected into thyroidectomized guinea pigs exactly as in the preceding experiment. The quantity of each preparation administered contained several times the amount of hormonal activity present in the stock extract which caused exophthalmos. Thus the amount of growth hormone or of prolactin administered daily was two to three times that contained in 40 mg. of the stock beef pituitary extract, even though the total amount of solids injected was much smaller.

If the exophthalmos resulted from the action of any one factor in the stock extract, it should also result when given in a more



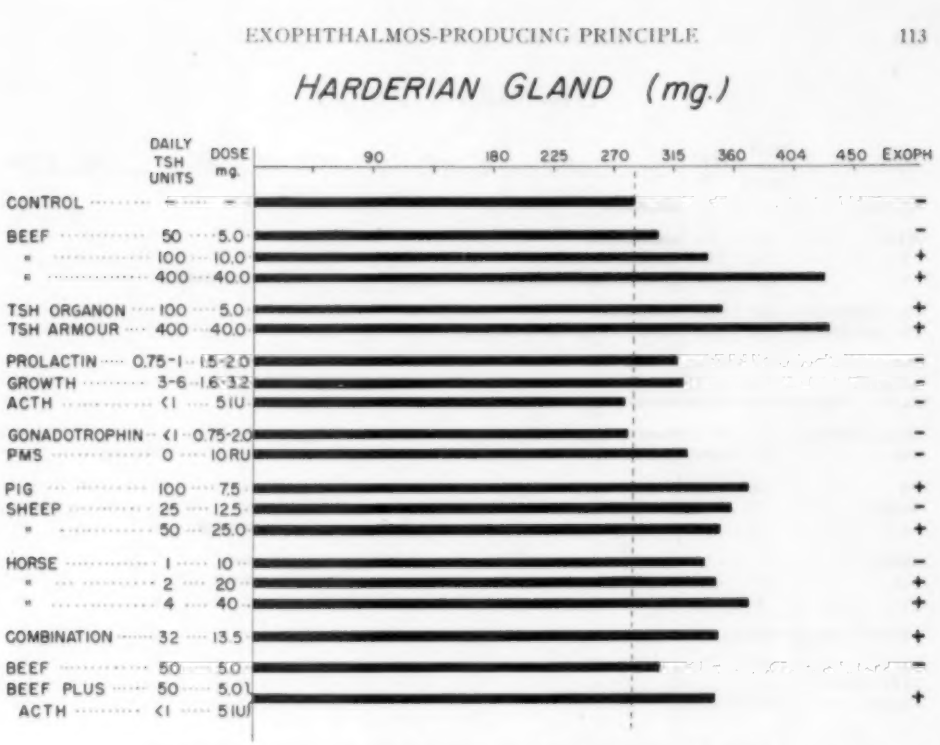
Graph 3 (Smelser and Ozanics). Studies on the exophthalmos-producing principle in pituitary extracts.

purified form. Since these preparations were not entirely pure, it was possible that exophthalmos could result from contaminants in them. In most preparations traces of thyrotropic hormone were present, but were determined by assay to be far less than the amount required to cause an exophthalmos, as shown in the first experiment.

Two preparations of thyrotropic hormone were also tested (thyrotropic hormone, Armour and thyrotropic hormone, Organon). Neither of these was pure, and their hormonal contaminants differed from each other and from the stock extract quantitatively and qualitatively. The quantity of each preparation injected was equal in thyrotropic potency to that of the stock, so that, if exophthalmos was due solely to thyrotropic hormone, it should be equal in degree, and the orbital modification should be identical in all experiments, whether the thyrotropic hormone had been in the form of a stock extract, Armour's, or the more potent Organon thyrotropic hormone.

If, however, exophthalmos was caused or modified by some contaminant in the preparations, it could very well be expected to vary in concentration in the three extracts tested, and the degree of proptosis could therefore be different.

Injection of extracts, each with a single predominant activity, showed that only the thyrotropic preparations produced exophthalmos. The amount of physiologic activity of growth hormone and prolactin, which were given as purified preparations, was 7.5 and 2.0 times, respectively, greater than that included in 40 mg. of the less purified stock material, yet no exophthalmos was produced with these extracts. There was some indication of a slight hypertrophy of the Harderian gland, but perhaps the thyrotropic contamination of the preparations could account for part of this.



Graph 4 (Smelser and Ozanics). Studies on the exophthalmos-producing principle in pituitary extracts.

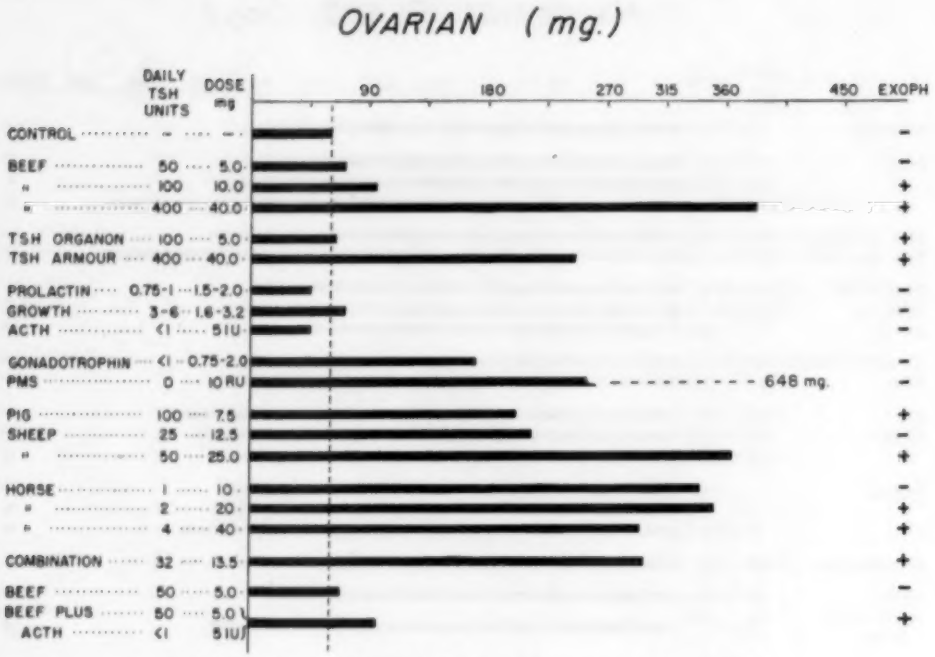
Neither did gonadotropic extract produce the slightest exophthalmos or hypertrophy of the orbital fat, muscles, or Harderian gland. Somewhat less ovarian hypertrophy was obtained in the guinea pigs than was expected on the basis of the assays conducted on hypophysectomized rats and immature mice. However, the ovarian and uterine response of the guinea pigs to gonadotropin (luteinizing hormone and follicle-stimulating hormone) was far greater than that caused by the gonadotropic activity contained in 10 mg. of the stock preparation or in the Organon thyrotropic hormone, both of which produced good exophthalmos and changes in orbital tissue.

Although the gonadotropin of pregnant mares' serum (PMS) is not of hypophyseal origin, a small group of animals was treated with this material. The ovarian response was very great, but no orbital changes occurred. The animals in this series were unfortunately larger than in the others, hence the greater values in some cases.

Follicle-stimulating hormone (FSH Armour) was also tested, although the results are not shown in the graphs. No exophthalmos or orbital changes were obtained, though marked ovarian and uterine hypertrophy did occur.

A number of ACTH preparations were used, but great difficulty was had in obtaining one capable of causing as great adrenal hypertrophy as resulted from the injection of 40 mg. Armour's thyrotropic hormone in the guinea pig, although they were very active by other tests. Many of these pituitary preparations, when injected in large amounts, caused some local toxic reaction.

The preparation finally used was administered in gelatine rather than in saline. It equalled the ACTH action of the stock prep-



Graph 5 (Smelser and Ozanics). Studies on the exophthalmos-producing principle in pituitary extracts.

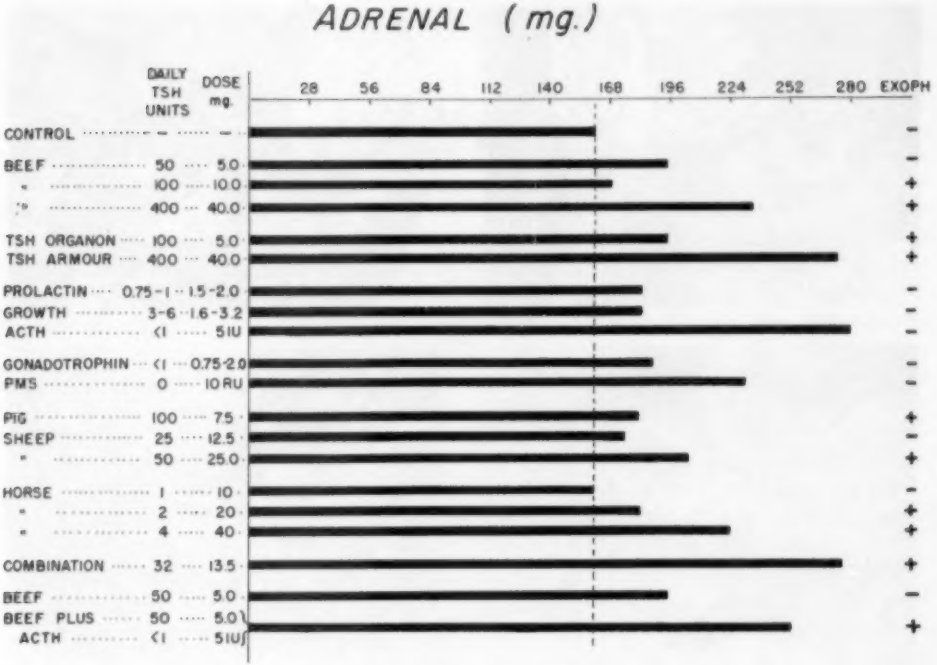
aration when tested in hypophysectomized rats. Daily injection of 5.0 I.U. did not produce the slightest indication of an exophthalmos, or modification of orbital contents (figure 1). However, adrenal hypertrophy of these animals was greater than that caused by the larger dose of stock extract, and far surpassed that provoked by 10 mg. of the stock or by the Organon thyrotropic hormone, both of which caused marked exophthalmos.

It seems clear, therefore, that exophthalmos and the associated changes are not the result of the action of any single one of the anterior pituitary hormones tested other than, possibly, the thyrotropic. Furthermore, *marked* ACTH or gonadotropic activity is not a necessary component of the exophthalmic producing extracts, as shown by the equivalent exophthalmos produced when equal amounts of thyrotropic hormone were injected, even though they were associated with variable quantities of ACTH and gonadotropic hormones. This is shown by comparison of the effects of the stock extract and of Organon and Armour's thyrotropic hormone (graphs 1 to 6).

III. Action of a combination of anterior pituitary hormones

Even though single hormones other than thyrotropic hormone appear to be without effect in the production of exophthalmos, it is conceivable that when present together they may act synergistically in this respect. It was decided to test this assumption by treating a series of guinea pigs with a combination of the same hormones as used in the preceding experiment, omitting of course, thyrotropic hormone.

The amount of each hormone (for example, gonadotropin, FSH, ACTH, prolactin, and growth hormone) given was greater than that contained in the stock ex-


ADRENAL (mg.)
DAILY TSH UNITS
DOSE mg.
28 56 84 112 140 168 196 224 252 280 EXOPH
CONTROL – – –
BEEF 50 5.0 –
" 100 10.0 +
" 400 40.0 +
TSH ORGANON 100 5.0 +
TSH ARMOUR 400 40.0 +
PROLACTIN 0.75-1 1.5-2.0 –
GROWTH 3-6 1.6-3.2 –
ACTH <1 5 IU –
GONADOTROPHIN <1 0.75-2.0 –
PMS 0 10 RU –
PIG 100 7.5 +
SHEEP 25 12.5 –
" 50 25.0 +
HORSE 1 10 –
" 2 20 +
" 4 40 +
COMBINATION 32 13.5 +
BEEF 50 5.0 –
BEEF PLUS ACTH 50 5.0 5 IU +

Graph 6 (Smelser and Ozanics). Studies on the exophthalmos-producing principle in pituitary extracts.

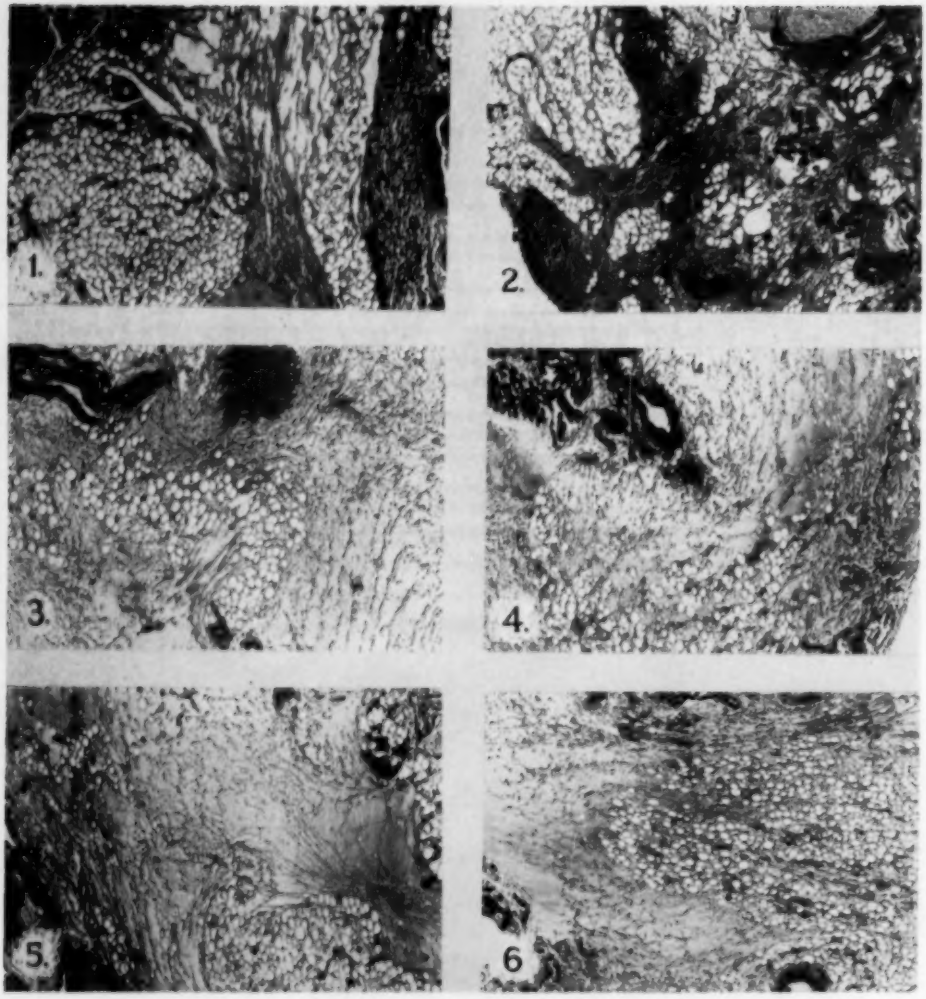
tract, with the exception of the thyrotropic hormone. Small amounts of this hormone were present, as a contaminant of several of the components. Due to the fact that these preparations formed a precipitate when mixed in a single solution they were injected into each experimental animal separately.

Six guinea pigs were injected with this combination of hormones in the same manner as in the other experiments. At autopsy four of them were found to be free of thyroid tissue and were definitely exophthalmic; the orbital fat and the Harderian glands were hypertrophied and the orbital fat was edematous (fig. 5). The results of the autopsy are shown in graphs 1 to 6.

The same combination of hormones was also assayed for its thyrotropic hormone content in chicks, and it was found that only 32 units of thyroid-stimulating material had been injected into the guinea pigs daily.

The combination of hormones contained a considerable quantity of ACTH, as may be judged from their effect on the adrenal weights of the test guinea pigs. It is possible that the exophthalmos which these injections produced resulted from the combined action of the ACTH and the 32 units of thyrotropic hormone which were present as contaminants.

To test this hypothesis, seven guinea pigs were treated with 5.0 mg. daily of the stock beef pituitary extract plus 5.0 I.U. of ACTH. Although these stock extracts contained some ACTH, its concentration was quite low as indicated by the degree of adrenal hypertrophy produced in the guinea pigs by this preparation. If the production of exophthalmos by thyrotropic hormone is appreciably enhanced by an excess of ACTH, a definite exophthalmos should be expected to result from the injection of these two materials and



Figs. 1 to 6 (Smelser and Ozanics). *Sections of the orbital fat of thyroidectomized guinea pigs injected with various pituitary preparations.*

(Fig. 1). Injected with 5.0 I.U. ACTH per day for 12 days. There are only traces of edema in the connective tissue, such as are seen in the fat of untreated thyroidectomized animals.

(Fig. 2). Thyroidectomized but uninjected control guinea pig. Here an unusually large amount of connective tissue is shown. In exophthalmic animals the edema fluid is prone to collect between the connective tissue fibers. Small amounts are normally found in the orbital fat of uninjected thyroidectomized guinea pigs.

(Fig. 3). Injected with 40 mg. (400 units thyrotropic hormone [TSH]) of the stock beef pituitary extract. Note the extensive edematous infiltration of the orbital fat. Sections of the retractor bulbi muscles are shown.

(Fig. 4). Injected with 40 mg. (4.0 units thyrotropic hormone) of horse pituitary extract per day. Note the large quantity of fluid which has infiltered between the fat cells. Compare with Figure 3.

(Fig. 5). Injected with a combination of gonadotropin, FSH, ACTH, prolactin, and growth hormone. Thirty-two units of thyrotropic hormone were present in each daily injection as a contaminant of the above preparations. Marked edematous modification of the fat, comparable to that seen in Figure 3, is shown.

(Fig. 6). Injected with 25 mg. (100 units thyrotropic hormone) sheep pituitary extract per day.

would be easily seen by comparison with data obtained when 5.0 mg. of the stock extract was injected alone.

The simultaneous injection of this quantity of ACTH and 5.0 mg. of the beef extract produced definite exophthalmos, although alone neither preparation, in this dosage, was capable of producing such a change. Both the protrusion of the eyes and modification of the orbital content was definite, as can be seen in Graphs 1 to 6. It seems evident that a synergism exists between some substances contained in the stock beef extract and the ACTH, and therefore that exophthalmos results from the interaction of two or more principles.

IV. Production of exophthalmos by extracts of pituitary glands of several species

It is well known that the hypophyses of different species vary greatly in the amount and proportion of the several hormones which they contain.[20-22] Nature has thus provided a good experimental means for testing whether exophthalmos results from the action of a specific hormone or from a combination of hormones. If it results from the action of thyrotropic hormone alone, then the degree of exophthalmos produced by hypophyseal extracts from various species should be proportional to the quantity of thyrotropic hormone they contain. Should it result from some particular combination of hormones, this also might be indicated by differences in the hormonal constituents of the several extracts and their ability to produce exophthalmos.

Extracts were prepared from the pituitary glands of the horse, sheep, pig, and beef. The beef pituitary extract was the stock material referred to earlier in the paper. The same method of preparation was used in all cases.

The starting material was, in the case of pigs, acetone dried anterior lobes of the pituitary glands. Sheep and horse pituitary glands, however, were received packed in dry ice. One additional batch of horse pituitary glands was obtained fresh, then frozen in dry ice for transportation. All of the frozen glands were dehydrated with acetone under refrigeration, and a dry powder was prepared.

Considerable difficulty was found in separating the posterior and anterior lobes of the horse and sheep glands, consequently these extracts were undoubtedly contaminated with posterior pituitary factors. All of the extracts were carefully assayed for their thyrotropic hormone, ACTH, gonadotropic, and growth hormone content. The most complete tests were made of the thyrotropic factor; no assays for their content of prolactin were done. These assays, of course, indicate only the amount of hormone present in the extract, which may have been somewhat different from that contained in the fresh glands before the extraction procedure.

The pig pituitary extract contained slightly more thyroid-stimulating hormone than did the stock beef preparation, therefore, in the exophthalmos tests, a proportionally smaller amount (7.5 mg./day) of the pig extract was injected. Six guinea pigs were used in this test, four of which became definitely exophthalmic, and the orbital fat hypertrophied and edematous. Two additional animals were given 10 mg. of this extract per day which resulted in exophthalmos and orbital changes similar to those of the first group. The data are shown in Graphs 1 to 6. The response of these animals to the pig pituitary extract was not significantly different from that of the group which received the stock beef extract of equivalent thyrotropic potency.

The sheep hypophyseal extract contained distinctly less thyrotropic hormone than did the beef preparation. Repeated assays showed that a minimum of 0.5 mg. was required to produce a thyroid hypertrophy in the chick equal to that caused by 0.1 mg. of the stock beef extract.

Eight animals were used in the test of the exophthalmos promoting ability of the sheep extract. Two guinea pigs were injected with

12.5 mg. daily. One of these became exophthalmic with the usual orbital changes.

The second group of six animals were given 25 mg. of the sheep extract per day, which in terms of thyrotropic potency was equal to that given the animals receiving 5.0 mg. of the stock beef pituitary extract.

The exophthalmos-producing potency of the sheep extract was definitely greater than that of the beef when they were compared on a basis of their thyrotropic hormone content. A good exophthalmos and orbital modification (fig. 6) was caused by the injection of 50 thyrotropic-hormone units of the sheep extract, whereas little or no effect was caused by 50 thyrotropic-hormone units of the beef preparation.

The extract of the fourth species tested was prepared from the pituitary glands of the horse. The two preparations used contained extremely little thyrotropic hormone. It was found that 10 to 20 mg. of the horse pituitary extract contained no more thyrotropic hormone than was found in 0.1 mg. of the stock beef pituitary extract. This astounding difference, 100 to 200 times, in thyrotropic-hormone potency was checked by repeated assays.

Extracts of both lots of glands were assayed in several groups of chicks simultaneously with a standard thyrotropic preparation. These assays showed them to be extremely deficient in thyrotropic hormone, although they contained much gonadotropic hormone and considerable quantities of ACTH and growth promoting material. The difference in potency between the beef and horse preparations found by us was greater than that observed by Rowlands;[22] however, his assays were not as extensive as those in the present study, and an unequivocal positive thyroid response to the horse pituitary extract was not obtained with the highest dosage he used.

Both of the horse pituitary preparations were quite toxic, and caused local reactions at the injection site when large doses were used. This was thought to be due to their contamination with the posterior pituitary principles. Extracts of posterior pituitary gland have been shown previously to be without effect in producing exophthalmos or changes in the orbital contents.

Although decidedly deficient in thyrotropic hormone, both horse pituitary preparations caused marked exophthalmos when injected into the test guinea pigs. Twenty mg. caused approximately the same degree of proptosis as did 10 mg. of the beef extract. The larger dose of the horse pituitary material (40 mg.) was only slightly less effective than the same quantity of either the beef stock extract or the Armour's thyrotropic-hormone preparation, even though it contained only one-one hundredth as much thyrotropic hormone. The orbital contents of these cases were hypertrophied, and the orbital fat edematous (fig. 4).

Discussion

In these experiments there was no clear dissociation of the several orbital changes which together cause the exophthalmos. Hypertrophy of the Harderian gland and orbital fat and its accompanying edema always occurred together. In only one group were orbital fat changes found without Harderian gland hypertrophy, and the reverse was never true. The reaction of these two tissues was not always precisely proportional, but variation due to the small number of cases may be responsible for this.

Marked hypertrophy of the extraocular muscles apparently occurs less readily than do the other manifestations, for when low dosages of thyrotropic-hormone were given, very slight or no change in weight of the extraocular muscle resulted. This is not surprising, for it has been shown that part of the muscle hypertrophy in exophthalmos is the result of the exophthalmos and not its cause.[13]

The experiments reported in this paper indicate that the power of pituitary extracts to produce exophthalmos is not necessarily proportional to their ability to induce thy-

roid-hypertrophy in chicks, that is, to their thyrotropic hormone content. However, the exophthalmic-producing factor is obviously much more closely associated with thyrotropic hormone than with any other single principle, for example, follicle-stimulating hormone, ACTH, gonadotropin, prolactin or the growth hormone.

Administration of each of these separately failed to produce exophthalmos, even when they were given in quantities four to 16 times that contained in the cruder extracts which did produce a proptosis. The preparations tested were not physiologically pure and presumably any of them could have contained, as a contaminant, a separate exophthalmos-producing material. Such a hypothetical factor, however, was not present in any of these extracts in amounts adequate to produce exophthalmos.

It would seem, therefore, that such a factor, if not the thyroid-stimulating hormone, is very similar to it, for it was present in each of several thyrotropic preparations (beef and pig pituitary extract, and the thyrotropins of Armour and Organon) which were used. In these cases the exophthalmos-producing potency was directly proportional to the thyrotropic-hormone content.

In addition, the chemical preparation necessary to obtain a high concentration of hypophyseal principles, even in a partially purified form, did not concentrate the exophthalmos factor except in the thyrotropic fraction. It therefore would appear that the thyrotropic hormone and the exophthalmos factor are at least closely related or chemically similar.

If similarity or close association are accepted, identity of the thyrotropic and exophthalmos factors appears to be denied by the experiments with sheep and horse pituitary extracts. Injection of 25 mg. of the sheep preparation produced a definite exophthalmos plus changes in the orbital tissues. Quantitatively this effect was far greater than that produced by 5.0 mg. of the similar beef preparation which contained the same thyrotropic activity of 25 mg. of sheep extract.

The experiments with horse pituitary extract are more striking because of the very low thyroid stimulating activity contained in these preparations. The degree of protrusion produced and orbital modification that accompanied it were marked, yet the thyrotropic hormone content of these extracts was only one percent that of the control beef extract which produced comparable ophthalmic effects.

Determination of the endocrine factors responsible for exophthalmos is handicapped by the lack of pituitary hormones in pure form. Furthermore, one can not ascertain whether or not the thyrotropic principles of different species are identical. The interstitial cell stimulating hormone obtained from the pituitary glands of pigs and sheep is apparently different. It is possible that the thyrotropic hormone obtained from the horse and beef also differ. Perhaps certain portions of the thyrotropin molecules are responsible for exophthalmos and others for the effect on the thyroid gland. Should this be the case, the proportion of these groupings may vary in different species. Our assays of thyrotropic hormone determine only its effect on the thyroid gland, leaving obscure other effects the thyrotropin molecule may have.

The result of the experiments with pituitary hormone combinations also does not support the idea that the thyrotropic hormone is exclusively responsible for exophthalmos. The degree of proptosis produced when the combined treatment was given was marked, and the total thyrotropic hormone administered was too small to have been alone responsible for it. This may be seen by comparing the effect of 32 thyrotropic-hormone units contained in the combination with the effect of 50 thyrotropic-hormone units contained in the beef extract.

If the production of exophthalmos by the injection of ACTH and 5.0 mg. of the beef extract was due to a synergism between

ACTH and thyrotropic hormone, then the 32 units of thyrotropic hormone plus the ACTH contained in the combination treatment could well be responsible for the exophthalmos produced in this experiment.

Since the beef pituitary extract which was used contained many hormones other than thyrotropic hormone, in addition to unknown substances, one can not determine whether this result was due to an interaction of ACTH with thyrotropic hormone or with other hormones known to be present. A synergism between ACTH and thyrotropic hormone seems to be the most probable at this time. With the above reservation these experiments confirm the observation of Aterman.[11]

In an earlier study[7] pituitary extracts were combined with adrenal steroids. Such cases were among the most markedly exophthalmic obtained, but since the quantity of pituitary extract used was large, any augmentation caused by the steroids was masked.

In experiments where exophthalmos resulted from the injection of extracts containing very little thyrotropic hormone (hormone combinations, horse, and sheep), considerable quantities of ACTH were present. This also suggests that ACTH may enhance the exophthalmic-producing power of thyrotropic hormone.

It is therefore tempting to explain the effectiveness of the sheep and horse extract on this basis. Both contained appreciable quantities of ACTH as well as gonadotropins and some growth hormone. However, it is difficult to believe that a synergism between ACTH and thyrotropic hormone is so powerful that the results of the horse and sheep experiment could be explained on this basis.

The quantity of ACTH contained in these extracts was less than the equivalent of 5.0 I.U. which, with 5.0 mg. (50 thyrotropic-hormone units) of the beef extract, produced a comparable exophthalmos. Furthermore the quantity of thyrotropic hormone in the horse pituitary extract was far less (4.0 thyrotropic-hormone units) than that contained in 5.0 mg. of the beef preparation (50 thyrotropic-hormone units).

It does not seem probable that 5.0 I.U. ACTH plus 4.0 thyrotropic-hormone units would cause an exophthalmos of the degree obtained in the present experiments. It would therefore appear that a third factor may be involved in the production of exophthalmos. However, no data are provided here which could decided this point.

Thyrotropic hormone may not be excluded from a role in the production of exophthalmos unless it should be found that a pure thyrotropic preparation fails to induce an orbital protrusion, even when given in combination with other hormones. There is no indication that ACTH itself has any exophthalmos-producing ability. It may act only in a permissive role, in which case thyrotropic hormone would be ineffective in the production of the proptosis in the absence of ACTH or its consequent adrenal steroids.

Summary

1. The injection of crude pituitary extracts containing a mixture of the well defined hypophyseal hormones causes exophthalmos in thyroidectomized guinea pigs. The degree of protrusion varies with the quantity of pituitary extracts given.

2. The protrusion obtained is not caused by gonadotropin (LH and FSH), FSH, ACTH, prolactin, or growth hormone when these extracts are injected separately.

3. Although hypophyseal extracts of various species containing thyrotropic hormone cause ocular protrusion, the exophthalmos-producing potency is not proportional to the thyrotropic-hormone content. Preparations of equal exophthalmos-producing potency may contain as little as one percent of the thyrotropic activity of the other.

4. The injection of a combination of hormone perparations (gonadotropins, FSH, ACTH, prolactin, and growth hormone) produces a definite exophthalmos, whereas these preparations fail to do so when injected

alone. Some thyrotropic hormone was present as a contaminant in these extracts, but its total concentration was too low to have been responsible for the proptosis.

5. The administration of ACTH in conjunction with a small quantity of pituitary extract causes a marked exophthalmos, whereas when injected separately in the same quantity neither cause this effect. Exophthalmos therefore appears to result from the synergistic action of two or more substances.

630 West 168th Street (32).

We are greatly indebted to Dr. Irby Bunding of the Armour Research Laboratories for the generous supply of pituitary material. It is a great pleasure to express our thanks for his co-operation and the materials. The preparations were TSH 377157; LH and FSH 377242H (horse origin); FSH K45108R; growth hormone R377237; ACTH M10402R in a gelatine vehicle. The activity expressed in International Units is equivalent to the U.S.P. standard.

The prolactin preparation 71713 which had a prolactin potency of 20 to 25 I.U. per mg. was furnished by E. R. Squibb & Sons through the kindness of Dr. R. W. Bates.

Dr. K. W. Thompson of Organon, Inc., kindly provided us with a generous supply of thyrotropic material 17510 which was equivalent in potency to the U.S.P. standard.

An acetone dried powder prepared from the anterior hypophysis of pigs was obtained through the courtesy of Dr. David Klein from the Wilson Laboratories.

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Discussion

Dr. Albert M. Potts (Cleveland): I find quite impressive this volume of work and the tremendous effort that was put into this paper.

I would like to ask Dr. Smelser whether it might be possible to eliminate some of his possibilities by using one of the small molecule ACTH preparations, such as Li has produced, so that at least one could be free of other protein contaminants and could do a chemical separation between large and small molecules.

Dr. George K. Smelser (New York): I think that is a very good suggestion. It has been made before. I don't believe we have any problem at the moment in deciding whether the ACTH is a potentiator or not. We have a good deal of difficulty in deciding whether the potentiated material is thyrotropic or not. When one obtains sufficient quantities of pure TSH so that such studies can be done, they will be done, either by us or some others interested in it.

As far as this particular study is concerned, ACTH was quite pure; however, it was ACTH protein and not ACTH polypeptide. Of the two polypeptide materials which Li has obtained, the one which affects adrenal hypertrophy rather than the one which causes the ascorbic acid to fall, is the one involved here.

We might point out also that if you have in your hands in a laboratory an absolutely pure thyrotropic material, you could not decide now whether it would cause exophthalmos or not unless you combined it with other pure preparations such as ACTH to find out if the exophthalmos is indeed not the result of the potential action of two hormones or possibly a third.

THE INFLUENCE OF ACTH AND CORTISONE ON THE REACTIVITY OF THE TERMINAL VASCULAR BED IN THE EYE*

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New York

In recent years some study has been made of the regulatory effects of adrenocortical secretions on the general circulation, the local blood flow, and the maintenance of vascular tone. Research studies have been conducted on both adrenalectomized and "hyperadrenal" animals, on patients with adrenal insufficiency and with general hypertension, and on normal subjects. The terminal vasculature in the mesappendix of the rat, in the rabbit ear chamber, and in the hamster's cheek pouch served as test material in the experimental work on animals; in a study of human eyes, A. Fritz (1951)[1b] described the functional responses of the retinal and optic nerve capillaries to various extraneous stimuli. Regrettably, neither the method of ophthalmodynamometry used by Fritz nor the reported experimental procedures of other investigators permitted quantitative analysis of the results.

In a previous study conducted in this laboratory (1952)[2] it was shown that minimal amounts of adrenaline and noradrenaline injected intravenously in cats elicited characteristic changes in the tracings of the intraocular pressure when a sufficiently sensitive method of manometry was employed. With a Sanborn electromanometer set at a high sensitivity range, measurable excursions of the writing stylus signified pressure changes of a small fraction of 1.0 mm. Hg.

The close dependency of variations of the

* From the Department of Ophthalmology, Columbia University College of Physicians and Surgeons, and the Institute of Ophthalmology, Presbyterian Hospital. This study was supported by the Knapp Memorial Foundation and the Snyder Ophthalmic Foundation.

eye pressure on minute changes in the blood pressure indicated that deflections in the eye pressure were caused by volume changes in the intraocular terminal vasculature. On the basis of this relationship, a method could be devised for the study of the behavior of the terminal vascular bed which excelled other techniques in sensitivity and fidelity of recording.

In the present study this method was applied to experiments designed for the examination of differences in the responses of the eye and blood pressures to exogenous noradrenaline, adrenaline, and acetylcholine, and to electric stimulation of the cervical sympathetic nerve, prior to and after continuous infusion of solutions of ACTH and cortisone. The cortisone was also administered by single subconjunctival or intravenous injection and, in other experiments, by repeated intramuscular injection. In addition, tentative experiments were performed on animals pretreated with desoxycorticosterone.

Materials and Methods

The experimental animals were young, mature cats weighing from 2.4 to 3.0 kg. General anesthesia was induced by intraperitoneal injection of chloralose in doses of from 55 to 70 mg./kg. Since the details of the experimental technique were described in a previous report, it will suffice to consider here only the improvements which were developed in the course of the current study.

In most of the experiments, artificial respiration was induced in the heparinized animals after the usual cannulization of the femoral artery for measurements of the blood pressure, and after preparation of the contralateral femoral vein for administration of the pharmacologic agent.

In one group of experiments the isolated cervical sympathicus nerve was placed on a fine electrode for electric stimulation by a square wave stimulator which could be varied accurately in output voltage and frequency. The platinum prongs of this electrode were four-mm. apart, and encased in a plastic chamber.

Trauma to the eyes was again minimized by the introduction of 30-gauge needles in the anterior chamber. The ink-writing kymographion of the earlier study was replaced by a third channel of the Sanborn apparatus to record the blood pressure. The timer units of the three channels were synchronized so that the comparison of changes in blood pressure and in the pressures of both eyes was less cumbersome than in the experiments with the endless belt kymographion.

For blood-pressure readings, a lower sensitivity range of the instrument was used; in these recordings a five-mm. excursion of the writing stylus corresponded to a pressure change of 25 mm. Hg as compared to the eye pressure recordings in which four mm. paper represented 1.0 mm. Hg.

The lyophilized preparation ACTHAR* in 25-mg. amounts was dissolved in from 25 to 100 cc. of a 0.9-percent solution of sodium chloride. A continuous drip infusion was used in early experiments. In later series, a Bowman Infusion Pump with a delivery rate of from 0.5 to 1.5 cc. per minute was substituted for this method as it allowed more accurate control of the dose; the infused volume was relative to the size of the animal and varied from 2.5 to 5.0 cc. per kg. On the average, between 2.5 mg. and 5.0 mg. ACTH per kg. were incorporated. This series consisted of 63 cat preparations.

To test the effect of the introduced volume of fluid on the eye and blood pressures, the isotonic sodium chloride solution alone was infused at an identical rate in a number of experiments. As a rule, the levoisomer of noradrenaline (bitartrate monohydrate salt)† and synthetic epinephrine bitartrate† were injected intravenously in doses of 0.1 and 0.2 μg. per kg., and acetylcholine chloride was injected in doses of 0.05 and 0.1 μg. per kg. Electric stimuli of 8 v., 20 F., 15 sec. fre-

* ACTHAR, Armour & Co.
† Winthrop-Stearn

quency usually gave satisfactory responses. The injections and the electric stimulations were repeated after the infusion of ACTH in the same sequence and with the same time-spacing.

In 10 experiments, the described infusion technique was employed with a water soluble cortisone preparation.* Suspensions of other cortisone compounds (commercial cortisone acetate,† microcrystalline cortisone acetate,‡ and microcrystalline free alcohol§) were injected intravenously in bulk, but the cannula in the femoral vein frequently became blocked and quantitation of the incorporated material was impossible.

In a series of 12 animals, cortisone acetate in doses ranging from 7.5 mg./0.3 cc. to 10.0 mg./0.4 cc. was injected subconjunctivally into one eye one day before the main experiment. The other eye received the same quantity of the suspension medium.

Finally, in four chronic experiments, attempts were made to produce a state of hypercorticism by daily intramuscular injections of 10 mg./kg. cortisone acetate on the three successive days preceding the measurements of responses to the adrenergic and cholinergic stimuli.

Six additional experiments were performed on animals pretreated for three days with 1.0 mg./kg. of desoxycorticosterone.

Results

Intravenous infusion of ACTH
(63 experiments)

As a rule, the amplitudes of the respiratory and pulse waves in the eye pressure tracings increased and the pulse frequency moderately decreased, at time intervals varying from three to 10 minutes after initiation of the intravenous infusion of ACTH in a 0.9-percent solution of sodium chloride. Analogous changes occurred in the blood pressure but were less marked by reason of the lower sensitivity ranges at which the record was obtained (fig. 1).

Blood and eye pressures remained either on the same level or, more often, displayed a flat rise during or following the infusion. Frequently, the total increment of the eye pressure was proportionally greater than that of the blood pressure, and the eye pressure tracing continued to rise after the blood pressure had reached a plateau.

Figure 2 shows such a rise of the eye pressure from 29 to 34 mm. Hg (O.S.) and 27 to 32 mm. Hg (O.D.) and of the blood pressure from 100 to 110 mm. Hg without accompanying increase in amplitude of the respiration waves. The dose of ACTH sufficient to produce the described responses varied with the animal; generally, 3.0 mg./kg. evoked the effect in sensitive preparations.

Adrenaline injected in a dose of 0.1 or 0.2 µg. per kg. usually caused in both pressure curves the characteristic biphasic response; the same dose of noradrenaline elicited a pure pressor reaction as previously described. The fall of blood pressure which followed the intravenous injection of 0.05 to 0.1 µg. per kg. of acetylcholine was reflected in the eye pressure by a deflection in the same direction, but this change was often preceded or followed by an elevation in the eye pressure which was independent of changes in the blood pressure (figs. 3 and 5). In three instances, the acetylcholine induced fall of the blood pressure was accompanied by a pure rise in the eye pressure (fig. 7).

Preganglionic stimulation of the cervical sympathetic nerve caused in 12 instances a response on the homolateral side which consisted of moderate increase of the eye pressure followed by a marked fall (fig. 8). The curves of the blood pressure and of the pressure of the second eye sometimes exhibited a slight synchronous rise. Variations of these

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* Solubilized cortisone T.C. (tricarballylate), Merck & Co.
† Hydroxycorticosterone-21-acetate, Merck & Co.
‡ Merck & Co.
§ Merck & Co.

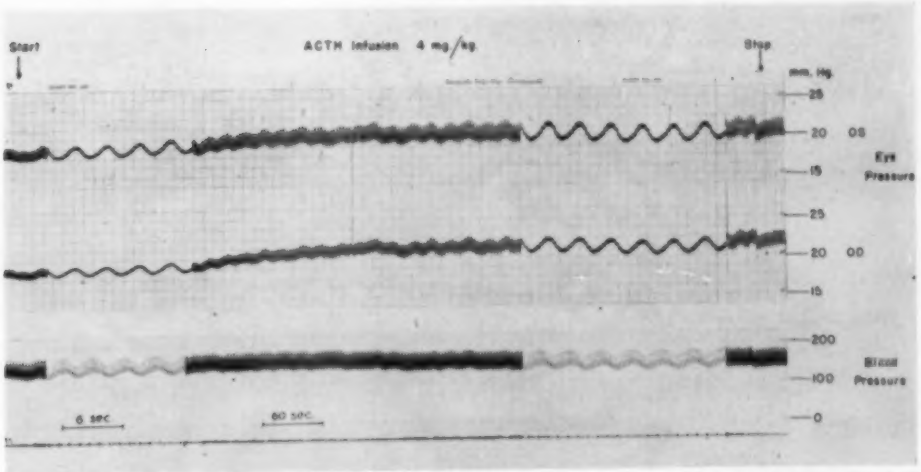


Fig. 1 (von Sallmann, Pillat, and Powers). Changes (Type 1) in characteristics of intraocular and blood pressures during ACTH infusion (4.0 mg./kg.). Low-speed and high-speed recording.

responses were noted in five experiments in which the pressure of the experimental eye showed only an elevation (fig. 9) or a depression.

A comparison of the pressure changes induced by the adrenergic and cholinergic agents and by electric sitmulation of the cervical sympathetic nerve, prior to and following the infusion of ACTH, showed in both pressure curves a potentiation of the effects of these stimuli by the treatment with ACTH. In 29 of the preparations, the increase in reactivity was pronounced; in 10, the increase under identical conditions was definite but of a lower magnitude; in the remaining 24, the responses were unenhanced.*

The relative degree of sensitization for individual stimuli varied from experiment to experiment within narrow limits. As Figures 3, 4, 5, and 6 illustrate, the augmentation of the studied effects was demonstrated by greater amplitude of deflection as well as by prolongation of the action.

In this sensitive preparation, the drug-induced increments of the eye pressure responses in the higher dose were 5.0 mm. Hg for adrenaline, 3.0 mm. Hg for noradrenaline, and 2.0 mm. Hg for acetylcholine. In relation to the readings prior to the infusion, ACTH doubled the effect of the catechols, and increased the response to acetylcholine by one third (figs. 5 and 6). Not in all experiments with marked responses were these pressure changes as pronounced, but reponses in this group were increased in a range of from 1.0 mm. to 5.0 mm. Hg for the eye pressure and from 10 mm. to 25 mm. Hg for the blood pressure.

The duration of the responses was about doubled in the case of noradrenaline (30 seconds to 50 seconds) and acetylcholine (40 seconds to 60 seconds); in the majority of experiments, ACTH prolonged the action of adrenaline only to a moderate degree.

In one instance, ACTH was infused into a preparation which had shown, following the injection of acetylcholine, a pure rise in intraocular pressure; after the administration of the hormone, the eye pressure tracing exhibited the normal response to acetylcholine (fig. 7). In several experiments, deflections in the tracings of the eye pressure

* It should be noted that included in this latter group are results obtained in the early part of the work when technical difficulties were frequently encountered.

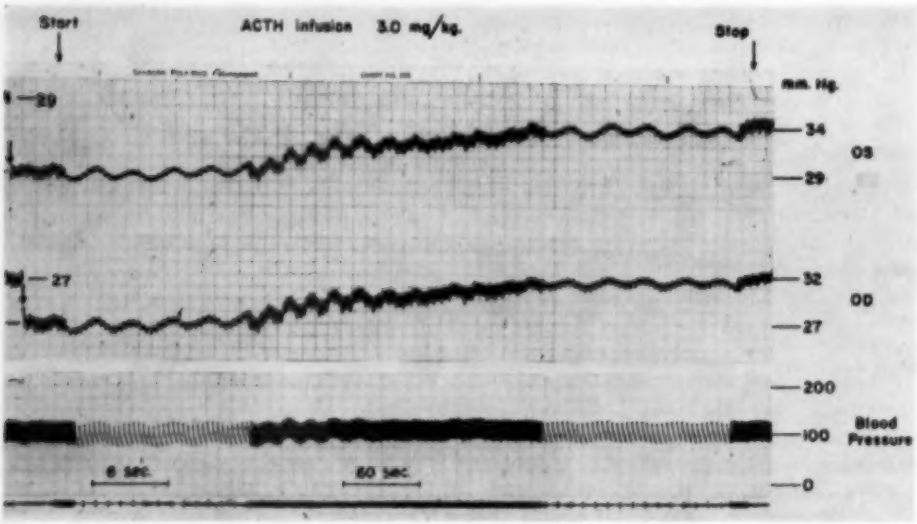


Fig. 2 (von Sallmann, Pillat, and Powers). Changes (Type 2) in characteristics of intraocular and blood pressures during ACTH infusion (3.0 mg./kg.). Low-speed and high-speed recording.

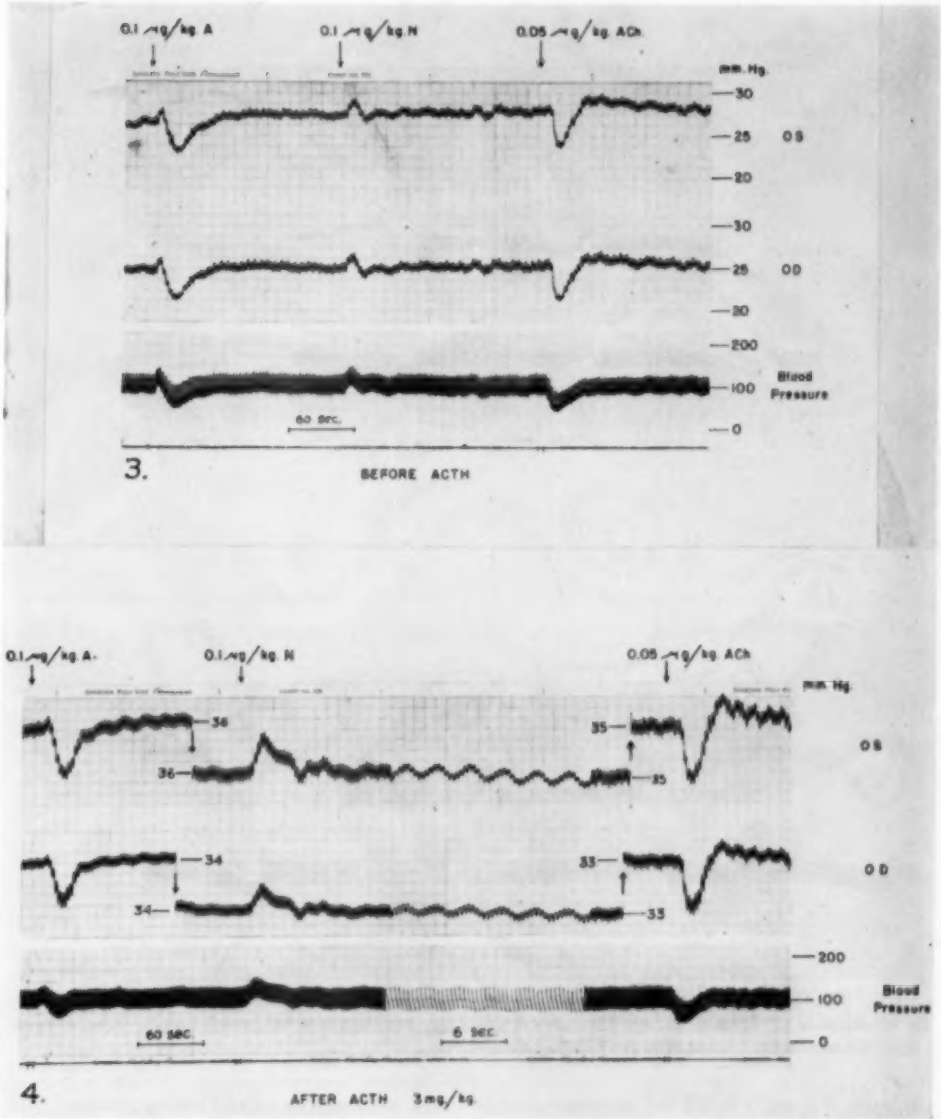
which were increased following ACTH were proportionally greater than the corresponding changes observed in the blood-pressure curves.

The state of hyper-reactivity persisted for from 30 minutes to one hour. The extent of the increase and the duration of the effect seemed to depend more on the sensitivity of the preparation than on the amount of ACTH or the incorporated volume of fluid. Control experiments with infusion of an equal amount of 0.9-percent solution of sodium chloride, or of mammalian Ringer solution, did not exhibit a sensitizing influence on the responses to the three types of stimuli. The amount of fluid (maximum 15 cc.) and the duration of the infusion (maximum 10 minutes) varied according to the size of the cat.

Intravenous injection of commercial cortisone acetate, (4 experiments), *microcrystalline cortisone acetate* (3 experiments), *and microcrystalline cortisone free alcohol* (5 experiments); *intravenous infusion of solubilized cortisone TC* (10 experiments); *intramuscular injection of cortisone acetate* (4 experiments); *and intramuscular injection of desoxycorticosterone acetate* (6 experiments)

It can be stated that in none of the 16 experiments in which cortisone suspension was administered systemically was there any evidence of a potentiation of the pressure effects of the studied stimuli. However, the low solubility of the preparations, the precarious nature of the method of administering them, and the time limit for the performance of the experiments precluded the drawing of any general conclusions as to the effects of cortisone on vasomotor responses.

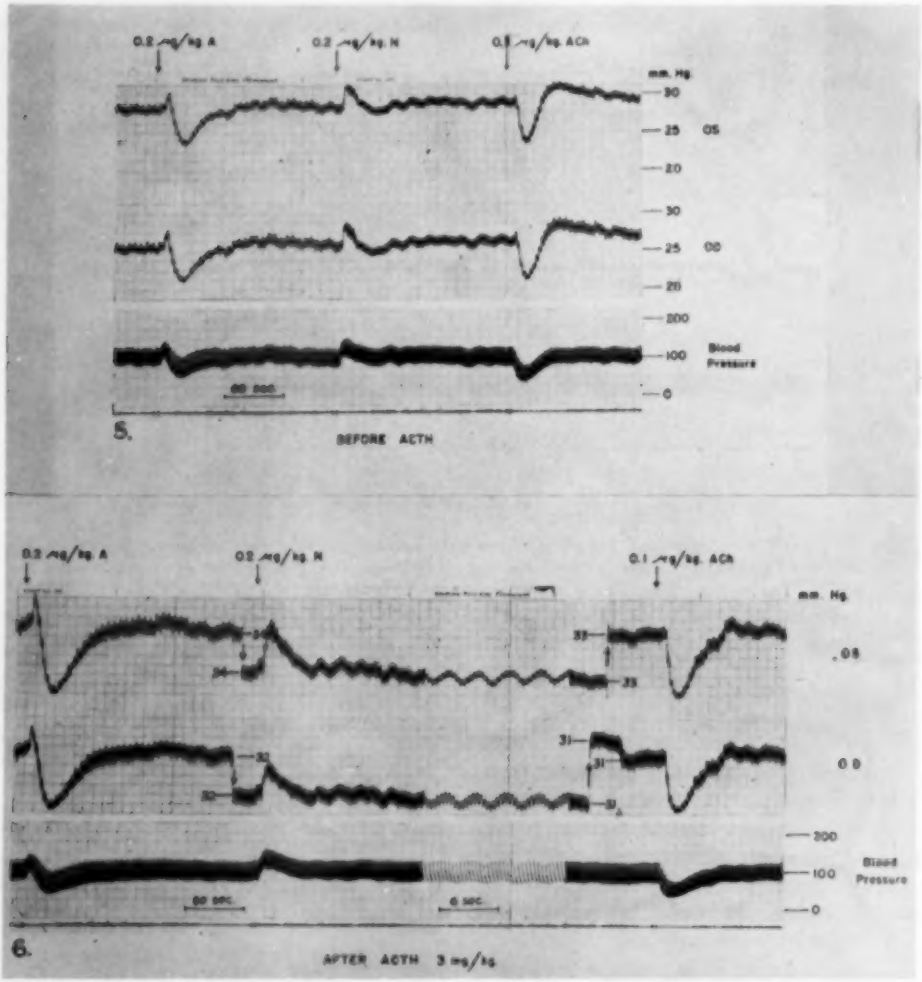
The infusion of the water-soluble compound held greater promise as an experimental procedure and provided conditions comparable to those that prevailed in the experiments with ACTH. In the 10 experiments of this group, in which 5.0 to 10 mg. of cortisone per kg. were incorporated, changes of the characteristics of the pressure curves did not occur and the responses to adrenaline, noradrenaline, acetylcholine, and electric stimulation remained unaltered. The animals were observed and tested up to



Figs. 3 and 4 (von Sallmann, Pillat, and Powers). Responses of intraocular pressure and blood pressure to intravenous injection of (A) 0.1 μg. adrenaline/kg., (N) 0.1 μg. noradrenaline/kg., and (ACh) 0.05 μg. acetylcholine/kg. prior to and after treatment with ACTH (3.0 mg./kg.).

one hour after termination of the infusion.

Experimental hypercorticalism produced by pretreating four cats with intramuscular injections of cortisone acetate did not permit an evaluation of the results in view of the great variations in the sensitivity of untreated cats to catecholamines. It was obvious, however, that the responses in these



Figs. 5 and 6 (von Sallmann, Pillat, and Powers). Responses of intraocular pressure and blood pressure to intravenous injection of 0.2 μg. adrenaline/kg., 0.2 μg. noradrenaline/kg., and 0.1 μg. acetylcholine/kg. prior to and after treatment with ACTH (3.0 mg./kg.).

animals did not exceed the responses often encountered in control experiments.

The effect of repeated injections of desoxycorticosterone acetate was studied on six cats which received 1.0 mg./kg. over a period of three days and on the experimental day. The pressure responses to adrenaline, noradrenaline, and acetylcholine in doses of 0.1 to 0.2 mg./kg. and to electrical stimulation were measured and were compared with the average pressure responses of normal animals. They were found to be of the same magnitude. On the other hand, the respiratory and pulse waves appeared to be increased. High amplitudes of these waves were a consistent characteristic of the curves obtained on the desoxycorticosterone-treated animals.

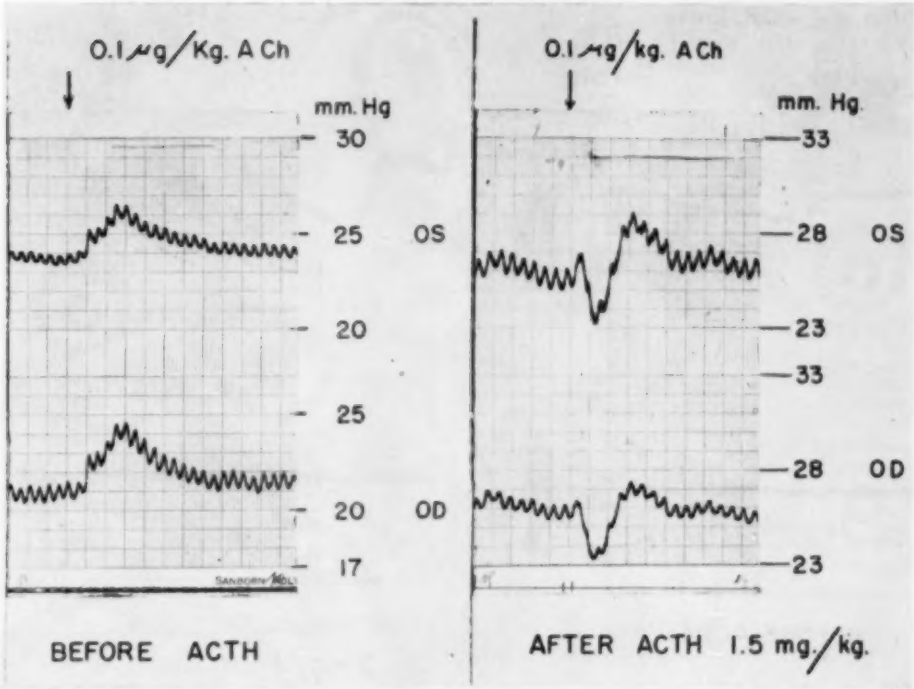


Fig. 7 (von Sallmann, Pillatt, and Powers). Abnormal pressor effect of acetylcholine (0.1 µg./kg.) on intraocular pressure; reversal of this effect following the infusion of ACTH (1.5 mg./kg.).

Subconjunctival injections of cortisone acetate (13 experiments)

In 13 experiments, one eye of each animal received subconjunctivally a dose of from 7.5 to 10.0 mg. cortisone acetate in suspension. In these eyes, the depot was visible on the following day. The other eye was injected with the same volume of the suspension medium (0.3 to 0.4 cc.) After a lapse of from 18 to 24 hours, the pressures of both eyes and the blood pressure were measured manometrically in the usual way.

In general, the levels of both eye pressures of the individual cat were found to be identical. Occasionally, slight differences in tension between the two eyes were observed; these differences did not exceed ±2.0 mm. Hg and are not a significant deviation from the values obtained in untreated preparations. In several experiments, the amplitude of the pulse and respiration waves was somewhat greater in the cortisone eye.

After adrenaline, noradrenaline, and acetylcholine had been injected intravenously in doses varying from 0.1 to 0.4 µg. per kg., an increase in the responses of the cortisone-pretreated eyes was suggested in eight experiments. In the remaining five animals of this group, no effects whatever were noted in the cortisone-pretreated eyes.

Comments

Although a review of the literature on the effects of adrenocortical steroids on vasomotor responses was for the most part unrewarding, with the data embodied in the published reports often conflicting, it seems appropriate to comment briefly on the experimental approaches selected by other investigators for the study of the influence of

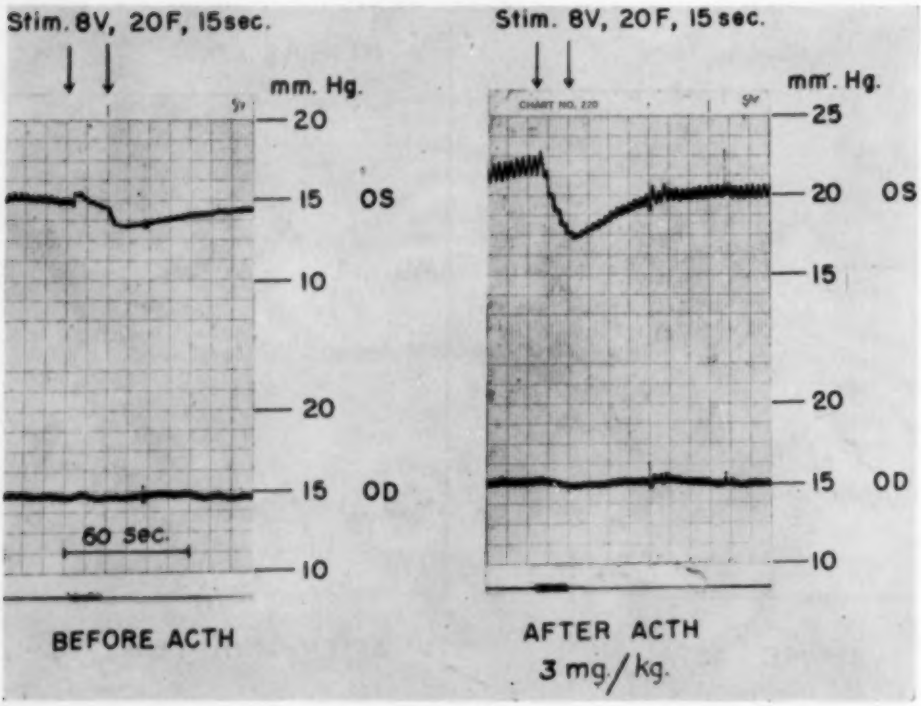


Fig. 8 (von Sallmann, Pillat, and Powers). Typical response of the intraocular pressure to electrical stimulation prior to and after treatment with ACTH.

these hormones on the behavior of the terminal vascular bed. The discussion will be restricted to the phenomena concerned with the present work.

To examine the effects of locally applied cortisone, Fritz (1950, 1951)[1a, 1b] devised three methods for estimating the capillary pressure in the optic nerve and retina of the human eye. By means of ophthalmodynamometry and a technique of indirect illumination, he looked for cortisone-induced changes in the color of the optic disc, in the filling of the retinal veins close to the margin of the optic papilla, and in the diameter of the retinal capillaries. Cortisone seemed to diminish and to stabilize the capillary pressure. It abolished the effects of parasympathetic pharmacologic stimuli and nullified the pressor response elicited by stimulation of the cervical sympathicus.

Contrarily, Kurland and Freedberg (1951)[3] noticed a striking potentiation of the blood pressure responses to noradrenaline in three normotensive human subjects who had been treated with cortisone.

On adrenalectomized dogs, Ramey, Goldstein, and Levine (1951)[4] observed potentiation of blood pressure responses to previously ineffective concentrations of noradrenaline as a result of single injections of adrenocortical extracts; normal control animals were unaffected by the same dose.

Ashton and Cook (1952)[5] in six experiments described a generalized vascular constriction and exaggerated tonic responses to extraneous stimuli in the vascularizing ear test chamber of the rabbit following treatment with intramuscular injections of 20 mg. cortisone acetate per day. They considered enhancement of the action of vasocon-

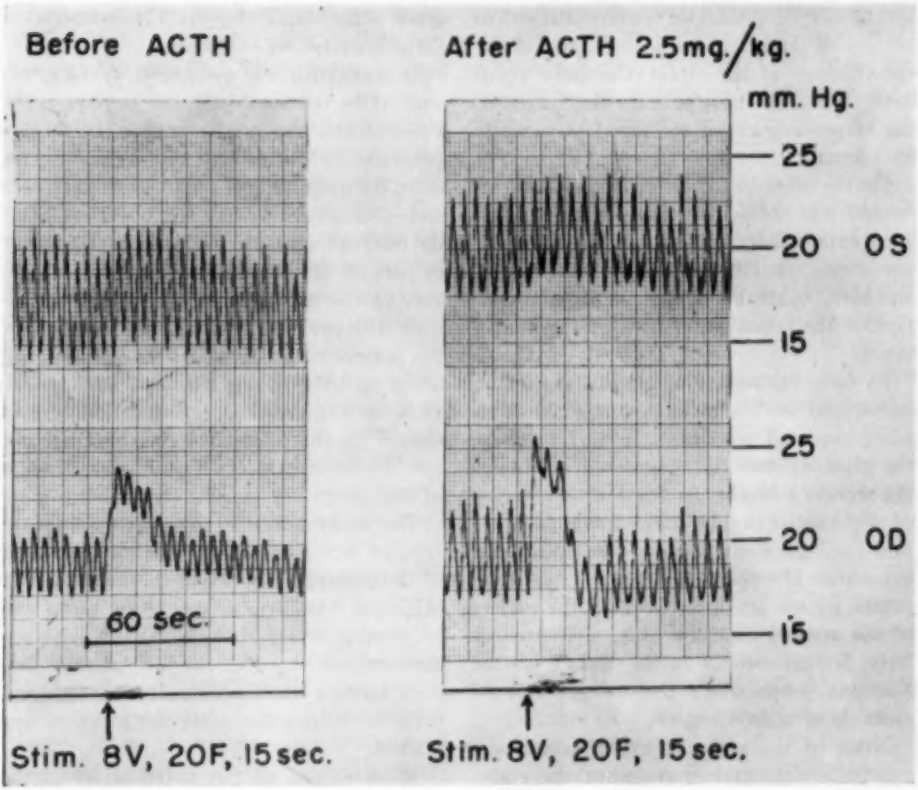


Fig. 9 (von Sallmann, Pillat, and Powers). Atypical response of the intraocular pressure to electrical stimulation prior to and after treatment with ACTH.

strictive agents by cortisone as the possible underlying mechanism.

Zweifach, Shorr, and Black (1952),[6] who studied the terminal vascular bed in the rat mesappendix, observed after adrenalectomy a progressive decrease of responses of the vessels to vasoconstrictive agents. The arterioles and venules gradually underwent dilation. With the administration of various adrenocortical steroids, the original reactivity of the vascular bed was restored. Stimulation of the adrenal cortex of normal rats by ACTH (200 to 600 μg.) was succeeded within two or three hours by increased vascular responsiveness to adrenaline, augmentation of vasomotion, and definite narrowing of the terminal arterioles and metarterioles. A similar array of changes could be produced in these preparations by the administration of large doses of cortisone. Acute or prolonged treatment with desoxycorticosterone did not influence the reactivity of the terminal vascular bed.

According to the observations of Wyman, Fulton, and Shulman (1953),[7] arterial vasoconstriction predominated in the cheek pouches of hamsters with experimental hypercorticism but there was an apparent decrease in the sensitivity to electric stimulation and to noradrenaline.

From these reports in the literature, it can be concluded that the administration of adrenocortical extract, or of ACTH, enhanced the effect of adrenergic compounds

and of electric stimuli on the terminal vascular bed of the rat's mesappendix and of the ear chamber of the rabbit. Cortisone treatment of normotensive patients also increased the responsiveness of the blood pressure to noradrenaline.

On the other hand, such a potentiating influence was not demonstrable in blood pressure experiments on normal dogs, in observations on the hamster's cheek pouch, and in the ophthalmodynamometric examination of the retinal capillaries of the human eye.

In fact, the results of clinical studies on the retinal vessels were in conflict with all other reported responses. In this instance, the great difficulties encountered in obtaining standard conditions for the observation of slight shifts in color may explain the aberrant findings. Contradictory results in other experimental approaches could have been caused by species differences, by the variety of the studied capillary beds, and, particularly, by differences in the nature of the hormonal preparations, the dosages, and the methods of administration.

None of the reported experimental procedures lent themselves to quantitative evaluation of the results except the observations on the general blood pressure. With the technique employed in the present study, measurements of the eye pressure showed this function to be extremely sensitive to volume changes of the intraocular vascular bed; in fact, such changes could be demonstrated graphically for fractions of a millimeter of Hg.

It was learned from the numerous experiments with ACTH that there were great variations in sensitivity among individual experimental animals. The speed and depth of the anesthesia, the state of the sympathetic tone, and the age of the cat may have accounted for some of these variations. Young cats in which chloralose anesthesia became effective only after a prolonged period post injection were often found to be the most sensitive preparations. In these instances, acute sensitization by ACTH infusion to various stimuli was striking.

In his extensive investigation, Wessely[8a, 8b] studied the relationship between eye pressure changes and the height of the intraocular pressure. In conformity with Wessely's results, the magnitude of the pressure increases and decreases which were observed after the intravenous use of ACTH was dependent in part on the level of the intraocular pressure of the experimental animal. Since animals with pressure readings above and below the normal range of from 18 to 24 mm. Hg were distributed uniformly among the various experimental groups, differences caused by the mentioned factor cancel out and do not interfere with the interpretation of the results.

The observations of Zweifach on the rat showed increased reactivity of the capillaries of the mesappendix two to three hours after injection, but, in our series, the pulse and respiration waves of the blood and eye pressure curves changed characteristically and their tendency to rise was already apparent three to 10 minutes after the infusion was started.

With regard to the potentiation of the adrenaline responses, the results were in agreement with Zweifach's; in addition, in our experiments the enhancive effect was also marked for noradrenaline. The influence of hypercorticism on cholinergic stimuli (physostigmine) has been mentioned in Fritz' study on the retinal vessels. Contrary to his findings, an increase in the sensitivity of the terminal vessels to acetylcholine was clearly demonstrated in the experiments on cats.

As to the type of change in the eye pressure following the injection of acetylcholine in small quantities, a fall in the curve was concomitant with and could be explained by a decrease in the blood pressure. A rise in the curve, either substituting for the fall, or more often preceding or following it, was independent of the blood pressure changes. To explain these opposing curves, it must

be assumed that the acetylcholine-induced dilation of the intraocular vascular bed overcompensated the effect of the lowered blood pressure.

The varying effects on the intraocular pressure caused by electric stimulation of the cervical sympathicus nerve were also augmented after the infusion of ACTH. The enhancement of reactivity, then, was not limited to adrenaline and noradrenaline but included the responses of preganglionic electric stimulation and the responses produced by small amounts of acetylcholine.

The results of the experiments in which cortisone was administered systemically by various routes were negative or extremely inconsistent. Zweifach's[6] experience with the rat's mesappendix was similar unless he used very large doses of cortisone and continued them until the day of the experiment. In our series the sensitivity of the cat preparation to the tested stimuli was unaffected by intravenous injections of various suspensions or the soluble compound, or by intramuscular injections in a series of chronic experiments.

The comparison of the drug-induced changes in the intraocular pressure in eyes pretreated locally with the cortisone emulsions and with the vehicle suggested, in the majority of experiments, that the cortisone had exhibited a minimal enhancing influence. The impression remained, however, that ACTH altered characteristically the reactivity of the terminal vascular bed in cats, and that cortisone did not. In the animals pretreated with desoxycorticosterone acetate, the character of the eye pressure tracings with consistently well-developed pulse and respiration waves seemed to indicate a regulation of vasomotoric responses by this compound.

Summary

1. Cat's blood and eye pressure records obtained with a sensitive method of electromanometry showed that intravenous infusion of from 2.5 to 8.0 mg./kg. ACTH potentiated, often dramatically, the reactivity of the peripheral vascular apparatus to adrenaline, noradrenaline, acetylcholine, and electric stimulation of the cervical sympathicus nerve.

2. This increased responsiveness to adrenergic, cholinergic, and electric stimuli began a few minutes after initiation of treatment and lasted for about one hour.

3. The characteristic influence of ACTH on the vasoresponsiveness of the cat preparation could not be duplicated in experiments in which cortisone emulsions were injected by various routes or in which a water-soluble preparation of cortisone was infused intravenously.

4. Experiments with desoxycorticosterone suggested that pretreatment with this preparation had a stabilizing effect on the vascular tone.

635 West 165th Street (32).

We are greatly indebted to Merck & Company for their co-operation in supplying us with the special cortisone preparations.

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QUANTITATIVE STUDIES OF BINOCULAR FUSION*

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The factors determining binocular fusion movements and the power of the fusion reflex have been particularly adamant to physiologic analysis.[1] Fusional compulsion is usually measured by creating a muscular disbalance either by means of a haploscope or by the prism vergence test.

"Fusion power alone, however, is probably not being measured, for in such tests at least three factors are involved: (a) The degree of forced convergence (or divergence); (b) the strength of the fusion compulsion reflex; and (c) the degree to which the otherwise normal accommodation-convergence reflex is altered or embarrassed."[2] In addition, marked fixation disparities are liable to occur and obscure the results when fusion is attained under conditions of forced vergence.[3]

Due to the psycho-optical nature of the fusion reflex,[4] very inconsistent results were also obtained when this reflex was investigated by diminishing the similarity of configurations conducive to binocular fusion or by reducing the illumination of binocularly presented identical fusion stimuli to near-threshold values.[5]

It was the purpose of the present investigation to attempt the elimination of these complicating factors by retaining the normal binocular stimuli of everyday vision, but merely varying the time element of the stimulus presentation. The time necessary for eliciting the fusion reflex was then taken as an index of the fusion compulsion. By examining a large number of subjects with apparently undisturbed binocular vision, information as to the range of the time element in normal binocular fusion was to be obtained.

METHOD

Figure 1 illustrates the experimental arrangement which is similar to that used in the study of the light tonus of the human eye.[6] In a well illuminated room (52 foot-candles) the observer looks with both eyes at a black screen (50 by 80 cm.) at a distance of six m. In the center of the screen is a small light source. White lines divide the screen into squares five cm. in length. The right eye can be occluded with a photographic shutter (Wollensak Alphax No. 2).

With an exposure time of 20 msec., the shutter can be released with frequencies varying from 20 sec. to one-fifth sec. In this manner only the interval between the exposures is changed while the duration of each exposure remains constant. In order to avoid vibrations the shutter is released by a 36-inch cable actuated by a solenoid. The frequency of the shutter opening is controlled by an electronic timing device which was designed for this particular purpose.

Fusion is tested by the presentation of the normal binocular fusion stimulus to the right eye in a series of brief flashes while vision with the left eye is unhindered. The test is based on the assumption that a low frequency exposure of the binocular visual field, for example, every 20 sec. for 20 msec., will not

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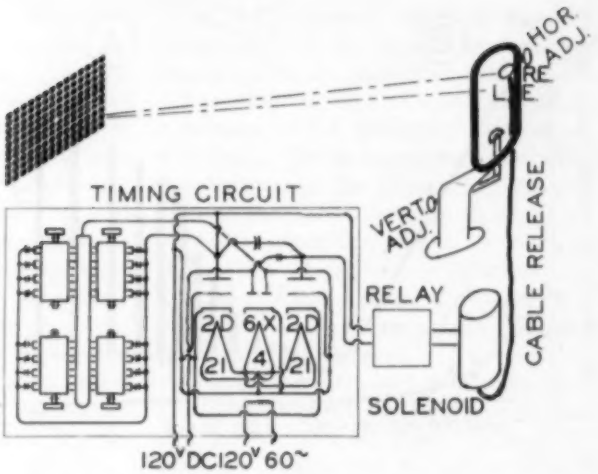

Fig. 1 (Brecher). Arrangement for measuring binocular fusion time. (See text for description.)

result in complete fusion, while more frequent stimulation, for example, every one-fifth sec., may bring about fusion due to a summative effect of the stimuli.

It is important that the duration of each exposure be less than the reflex time for the extraocular muscles or the period of retinal persistence. Only then the duration of the stimulus will have no direct effect upon the process of fusion. Twenty msec. was found to be the longest exposure time commensurate with this demand.

The visual field of the left eye comprises not only the screen but all contours of the room. During exposure the same is true for the right eye except that the peripheral field of vision beyond 50 degrees is permanently occluded by the shutter diaphragm. The stimulus evoking fusion is therefore provided by the identical contours of the practically unimpaired normal visual fields. Since in these experiments the entire visual fields act as fusion stimuli, the light source in the screen center is relatively unimportant as a fusion stimulus; it acts mainly as an indicator to measure subjectively the degree of diplopia or fusion.

Prior to each experiment the refractive status, heterophoria with the Maddox rod at a distance of six m., and eye dominance with a modified anaglyph test are determined. The subject is placed six m. in front of the screen and apparently undisturbed binocular fusion ability ascertained. Then the right eye is occluded for 20 sec. with the shutter, and upon shutter release of one-fiftieth sec. duration the subject is asked to report the position of the double image of the light source on the screen. Then the subject is tested in the same manner with varying frequencies of exposure.

The time necessary for attainment of complete fusion upon more frequent stimulation is called "fusion time." It is taken as an index of the compulsion for binocular fusion. The subject records this time with an automatic stopwatch. Thus 106 subjects (chiefly medical students) were examined and 13,765 individual readings taken.

Results

All subjects except three reported that the double images fuse when the right eye image is presented every one-fifth second. On the other hand nobody was able to accomplish binocular fusion when the exposure interval was 20 sec. or longer.

This is illustrated by the blocks in Figure 2-A. Each block represents the percent of subjects who failed to fuse at different

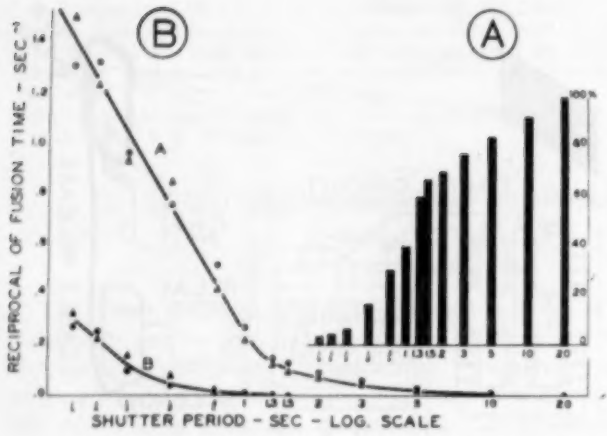

Fig. 2 (Brecher). Effect of stimulation frequency (abscissa) upon binocular fusion time (ordinate). (2-A) Percentage of subjects failing to accomplish binocular fusion at different exposure frequencies. (2-B) Fusion time of "normal" fusers (curve A). Fusion time of "slow" fusers (curve B).

frequencies of fusion stimulation. The values for the latter are found on the abscissa. It is noted by the increasing height of the blocks from left to right that most subjects failed to fuse when the interval between the stimuli became longer than one second. (The data of 11 subjects who reported fusion before the second exposure are not included in these figures; this group will be discussed later.)

Curves A and B in Figure 2-B are graphic representations for different groups of subjects of the time necessary to accomplish complete binocular fusion (fusion time) at different frequencies of the fusion stimuli. The reciprocal of the harmonic mean of the fusion time is plotted on the ordinate while the log of the stimulation frequency is plotted on the abscissa. The majority of the subjects (61) had a fusion time of 770 msec. (harmonic mean) ($\pm$39 msec.) at a stimulation frequency of five per sec. The fusion time of this group at different stimulation frequencies is represented by Curve A (open circles). It is noted that the fusion time became progressively longer when the stimulation frequency was decreased. From this it must be concluded that the time necessary for accomplishing binocular fusion is a direct function of the stimulation frequency.

A group of 11 observers displayed conspicuously long fusion times (over a second even with the fastest stimulation). This group of "slow fusers" is depicted by Curve B (solid circles) in Figure 2-B. It is noted that the trend of this curve does not differ from that of Curve A, except that a larger number of subjects in Group B failed to fuse at exposure frequencies of one sec. and longer.

Ten subjects showed orthophoria when their right eye was briefly exposed after 20-sec. occlusion. In order to test their fusion power an artificial esophoria was created by means of one to two prism diopters in front of the right eye. On the other hand, the heterophoria of 10 subjects exceeded the average values (98 minutes of arc horizontal without regard to sign) and the fusion they obtained at one-fifth sec. exposure frequency was inconsistent. An adequate prism was used in these cases to diminish the degree of heterophoria. With the use of such prisms they were able to accomplish fusion just as quickly as "normal" subjects. In fact, the fusion times at different stimulation frequencies of the 10 orthophoric and 10 marked heterophoric subjects did not significantly differ from one another nor from that of the "normal" subjects. Comparison of the values for the average fusion time of the

prism bearers (open triangles) and those represented in Curve A (open circles) of Figure 2 illustrates this finding.

In addition there were four prism bearers (two orthophoric and two marked heterophoric) whose fusion time lasted over two sec. at a stimulation frequency of one-fifth sec. These subjects are represented by solid triangles in Figure 2-B. Their average fusion times do not greatly differ from those of the 11 slow fusers in Curve B (full circles) without prisms. From this one may conclude that, within limits, the use of prisms for the creation or reduction of muscle imbalance does not seem to alter the fusion times significantly from those observed in subjects without prisms.

One group of 11 observers responded differently from the rest. They displayed about normal fusion times at one-fifth sec. stimulation but with less frequent stimulation their fusion time became shorter instead of longer. In fact, they reported fusion even before the second exposure occurred when the stimulation frequencies were in the range of one-half to five sec. This is illustrated in Figure 3.

Curve A is a plot of the reciprocal of the average mean fusion times of the "normal" subjects (averages of open circle and open triangle data of fig. 2-B). Curve B shows the slow fusers with and without prisms (averages of solid circles and solid triangles of fig. 2-B). Curve C represents the mean fusion times of the subjects who reported fusion before the second exposure.

The ascending limb of Curve C indicates a decrease of the fusion time with slower stimulation. The leveling of the curve shows the shortness of the fusion times reported, even at stimulation frequencies of 10 sec. However, when the interval between the stimulations was as long as 20 sec. all subjects of this group revealed dissociation of the images and were incapable of "holding their fusion" any longer.

Tentative data obtained with occlusion of the left instead of the right eye revealed that the eye used for fixing the target did not seem to influence the fusion time. In order to study the question whether dominance of one eye affects the fusion time, all subjects were tested with a modified anaglyph and the data correlated with the fusion time.

The insert in Figure 4 shows the pattern of the anaglyph consisting of red and green vertical lines separated by a black horizontal line. The subject who views this target through red (8.27 percent luminous transmission) and green (3.77-percent luminous transmission) filters must state whether the vertical lines above or below the horizontal appeared closer to him.

By alternately inverting the test object and interchanging the filters, four judgments

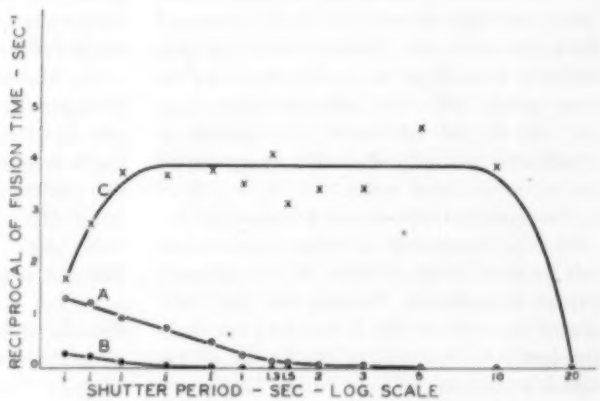

Fig. 3 (Brecher). Relation of fusion times of "normal" fusers (curve A), "slow" fusers (curve B), and subjects who fuse in the interval between first and second stimulus (curve C).

were recorded: (a) a response according to the light intensity difference of one and the other eye (23 out of 85); (b) images appear equally separated in spite of inequality of the filter transmission (19 out of 81); (c) alternating dominance (36 out of 81); (d) persistent dominance of one eye (right 5, left 4).

The graph in Figure 4 permits a comparison of the mean fusion times of the right and left dominant groups (Curve B, open circles with horizontal line) with those of the mean fusion time of all subjects (Curve A, open circles with points, average of all groups except those which fuse before the second stimulus).

It is noted that the fusion time values of the subjects with eye dominance at one-fifth sec. stimulation nearly equal those of the normal subjects. However, with larger stimulation intervals the former fused more slowly and finally failed to fuse altogether before the normals reached their limit as exemplified by the earlier drop of Curve B to the zero line. In these respects no difference in the behavior was noticed between right or left eye dominance.

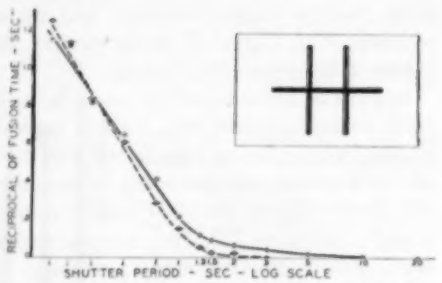

Fig. 4 (Brecher). Influence of eye dominance on fusion time. (Solid curve) Average of "normal" and "slow" fusers. (Broken curve) Subjects with right and left eye dominance. (Insert) Anaglyph pattern used for determining eye dominance.

Discussion

These experiments demonstrate that it is possible to obtain a quantitative estimate of the binocular fusion process by measuring the fusion time at different frequencies of stimulation. Making use of the normal binocular fusion stimuli the method reported here eliminates two factors which are persistently included in the measurement of fusion power with the prism-vergence test, (a) the degrees of forced convergence or divergence and (b) the embarrassment of the accommodation reflex.

The question now arises whether the fusion time measured in these experiments can be considered an index of the subject's fusion compulsion. Actually the test furnishes two criteria which one may use independently to characterize the degree of fusional attraction: (a) the fusion time measured at a given exposure frequency, such as at a stimulation every half second, (b) the fusion frequency at which a subject fails to fuse.

The two criteria are interrelated insofar as most subjects with a long fusion time at a given frequency are also the first ones who fail to fuse when the interval between the exposures is prolonged. This is illustrated by comparing the curves for the slow fusers with that of the normal fusers in Figure 2-B. Due to this correlation, either of the two criteria may be used as an index of the fusion compulsion.

No definite preference as to either criterion can be given yet from the present data. It appears, though, that the data obtained by measuring the fusion time at one given exposure frequency are more consistent than those determined by the stimulation frequency at which fusion fails.

On account of this finding, a simplified arrangement for measuring fusion compulsion can also be used by keeping the stimulation frequency constant. For this purpose, the photographic shutter is replaced by a small black rotating cardboard disc with three slots, about seven arc degres each. The disc is mounted on a small synchronous motor of one revolution per second. The constant stimulation frequency then permits comparison of the fusion time of different subjects.

Figure 5 shows a graphic presentation of the statistical analysis of the fusion time of 100 subjects at a constant stimulation frequency of three per second. According to this analysis, the average "normal fusion time" is approximately 1.2 seconds at an exposure of the visual fields three times per second.

Under two circumstances, the time required for fusion can only be measured by the introduction of an auxiliary prism; (a) if the subject's phoria is so small that no initial separation of the images is perceived after 20 sec. of monocular occlusion (orthophoria), or (b) if the phoria is so large that the most frequent stimulation of five per sec. fails to bring about complete fusion consistently. In either case, a prism was used to create or reduce the phoria for measurement purposes. This procedure seems to be valid since the fusion times of the prism bearers and nonprism bearers were in good agreement.

The subjects who consistently reported fusion before the second exposure occurred throw an interesting light on the fusion mechanism. This mechanism has two components, the motor and the sensory ones.[7-9] The motor component consists in the muscle activity for moving both eyes into such a position as to project similar images on corresponding Panum areas. The sensory component consists in the sensation of fusing the double images into one.

Ordinarily, both components run parallel and are also inseparable as far as time is concerned, because one can perceive the moving of double images toward one another, and when the motor action is accomplished, the images appear to be fused.[10] However, in the case of the subjects who report fusion within the interval between the first and second stimulus, the fusion time is apparently a measure of the sensory component only.

This may be explained as follows: If the phoria be small enough, no extraocular muscle movements (motor component) may

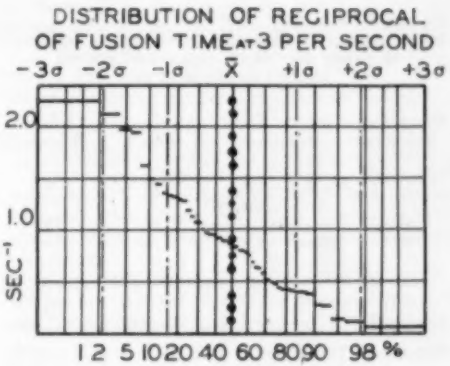

Fig. 5 (Brecher). Statistical analysis of the distribution of fusion time. Stimulation frequency constant: Three per sec. (Ordinate) Reciprocal of fusion time in seconds. (Abscissa) Percent of cases on normal Gaussian distribution scale. On this type of plot, a straight line signifies normal Gaussian distribution. "Normal Fusion Time" is about 1.2 sec. at three exposures of binocular visual fields per sec. (intersection of curve with central ordinate, dotted and marked *X*).

be required to bring the images on corresponding Panum areas. When the 20 msec. exposure occurs, the images appear separate, however, though very close, until the sensory fusion process has united them to one image. By that time, the brief exposure is over and the subject indicates the occurrence of the sensory fusion then by releasing the time recorder. When the next exposure occurs, the sensory fusion is still persisting and no image separation is noticed. Only when the interval between the stimulations exceeds 10 sec. the persistence of the sensory fusion seems to disappear (drop of right limb of curve C in fig. 3). The consistent shortness of the fusion time determined in these subjects may also indicate that the motor component of the fusion reflex is not being measured in these cases.

This explanation is supported by a survey of the data on phoria as shown in Table 1. It is noted that, without regard to sign, the average phorias of the group of subjects which showed fusion within the persistence of the first flash was only 15 minutes of arc horizontally and nine minutes of arc

TABLE 1

MEAN PHORIA COMPONENTS (REGARDLESS OF SIGN) OF THREE GROUPS OF OBSERVERS, PHORIA VALUES IN ARC MINUTES

Number of Subjects	Fusion Type of Subjects	Horizontal Phoria	Vertical Phoria
11	Fusion after first exposure	15	9
67	"Normal"	44	13
16	"Slow"	74	26

vertically. This measure is still within the extent of the Panum area which is, according to the reports of different authors,[2] between five and 35 minutes of arc horizontally. The average phorias of the normal subjects (group represented by curve A in fig. 3) is greater than the extent of Panum's areas and those of the slow fusers (curve B, fig. 3) by far the greatest.

In spite of the obvious statistical correlation of the degree of the average phoria and the fusional behavior, caution must be expressed as to the generalization that large heterophorias must be associated with slow fusion because numerous exceptions were observed during the experiments. Some subjects with large heterophorias fused very promptly whereas others with only small phorias were "poor fusers" displaying long fusion times. This finding indicates that fusional power as such is not necessarily dependent upon the degree of heterophoria, and that this new method permits an evaluation of the individual's power to fuse.

SUMMARY

A new method is described which permits the evaluation of an individual's binocular fusion power. The time required for accomplishing binocular fusion is determined in 106 subjects with apparently undisturbed binocular vision. The contours of a well-illuminated room are continuously presented to one eye, while the same visual field is presented to the other intermittently by occluding it and exposing it for 20 msec. at intervals varying from one-fifth to 20 sec.

Binocular fusion was obtained by all but three subjects at a stimulation of five per sec., while all persons failed to fuse when the interval between the stimuli was longer than 20 sec.

The fusion time measured is taken as an index of the compulsion for binocular fusion which is in this method uncomplicated by the introduction of forced vergence or an embarrassment of the accommodation convergence reflex.

The average fusion time of 100 subjects is about 1.2 seconds when the binocular visual fields are stimulated three times per second. Though subjects with large heterophorias generally have longer fusion times than those with small phorias, some persons with marked heterophorias are able to fuse very quickly, indicating that the power to fuse does not necessarily depend upon the degree of heterophoria.

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Discussion

Dr. Gerhard A. Brecher: May I just add that I did hope one question would come up in the discussion, but it was not openly voiced. The question is: How does this fusion time correlate with the degree of heterophoria observed in a person?

It does correlate to some degree. We have found that, generally speaking, the larger the heterophoria, the longer also the fusion time; and the smaller the heterophoria, the smaller the fusion time.

This is not always true, however. There are people who have marked degrees of heterophoria who are excellent and fast fusers, whereas, others with very small heterophorias are very slow and poor fusers.

This method, therefore, gives us the possibility, independent of the degree of heterophoria, to evaluate the fusion power.

LENS METABOLISM AS STUDIED WITH THE REVERSIBLE CATION SHIFT*

I. The role of glucose

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Portland, Oregon

When the rabbit lens is refrigerated at 0°C. (or subjected to certain other insults) it loses potassium, gains sodium, and becomes hydrated.[1] These changes can be reversed if the refrigerated lens is subsequently incubated at 37°C. Potassium reenters and sodium is excreted from the lens, the cation movement in both cases being against a concentration gradient.[2] Under appropriate conditions the normal cation distribution can be re-established. Dehydration of the lens also results.

The mechanism which controls the cation distribution is not known with certainty. However, for purposes of exposition the break-down illustrated in Figure 1 is sufficiently valid. At the limiting barrier (which may be the lens capsule, as well as the lens fiber membrane) is an active process which concentrates potassium within and sodium without the lens. (Implication is not intended that there is necessarily an active transfer of both sodium and potassium. The distribution may result from an active excretion of sodium alone, potassium entering passively to maintain electric neutrality.) In addition, this barrier permits a passive transfer, that is, a "leakage" of cations in the direction of the concentration gradient.

The two processes are perhaps not so independent of one another as such an account may indicate. Yet, there is justification for considering them separately.[3]

The steady-state ratio of the cation concentrations between the lens and external medium represents a balance of these two factors. Parenthetically, it should be noted that the type and concentration of organic anion determines to a certain extent the total base of the cell without necessarily influencing the potassium : sodium ratio.

One of the consistent lens changes ob-

* From the John E. Weeks Memorial Laboratory, Department of Ophthalmology, University of Oregon Medical School. Supported by a grant from the U. S. Public Health Service.

served during cataract formation is a shift in cation content toward equilibrium and a hydration of the lens. Thus a study of the reversal of the cold-induced lenticular hydration and cation shift seemed a logical approach to the problem of the pathogenesis of cataract and the reversal of cataractous change.

As these studies progressed, it became apparent that they permitted an evaluation of the effect of various factors on lens metabolism and function that other laboratory measurements, for example, glucose consumption, were incapable of providing. The present report is concerned with an investigation of the role of glucose in the reversal of the cold-induced cation shift.

Methods

The technique employed has been previously described.[3] The lenses were extracted from a pair of freshly enucleated rabbit eyes by turning back the sclera and cutting the zonules. Each lens was placed in a tube containing three ml. of the solution under investigation. The overlying air was displaced with a five-percent CO_2-95 percent O_2 mixture, the tube tightly stoppered and refrigerated at 0°C. for 41 to 45 hours.

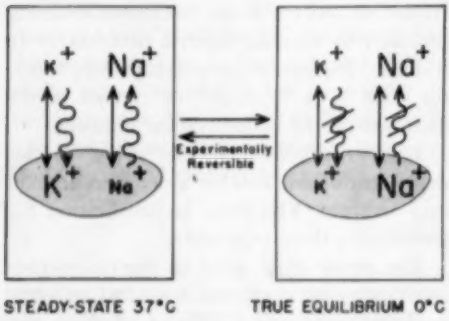

Fig. 1 (Harris, Hauschildt, and Nordquist). Schematic representation of control of cation content in the lens.

At the end of that time, one lens of each pair was analyzed immediately and the other was placed in a water bath at 37°C. for six hours. (The six-hour period was chosen because studies of the kinetics of the uptake of potassium and excretion of sodium following refrigeration have indicated that a steady-state is achieved at that time.[2])

For analysis, the lens was weighed rapidly, dried to constant weight at 105°C., and dry ashed at 480° to 500°C. with the aid of a small amount of concentrated sulfuric acid. The Baird Associates' flame-photometer was used for sodium and potassium determinations.

A modified Tyrode's solution containing more bicarbonate and potassium than the original formula specified was employed throughout.[2] Glucose and other substances were added as indicated. All solutions were adjusted to physiologic pH (7.32 to 7.48). The alterations in the pH during the procedure were minor and did not contribute a source of error. Sterile solutions and aseptic precautions were used throughout.

A minimum of eight pair of lenses was used in any particular study. Generally, results are expressed in terms of percent recovery* rather than as differences in cation concentrations between the refrigerated and the subsequently incubated lens. Percent recovery is the more accurate expression of the ability of the lens to return to normal, that is, to regain its original cation distribution. A statistical analysis has been employed wherever there was any question as to significance of a result.†

Shift of cations during refrigeration

An analysis is presented in Figures 2 and

* The formula is $\frac{(C_t\text{-}C_o)}{(C_i\text{-}C_o)} \times 100$ where C_t, C_o, and C_i are respectively the concentrations after refrigeration plus incubation, after refrigeration alone and normally observed.

† The t-test was employed to establish statistical significance, accepting $p = 0.05$ as the critical level.

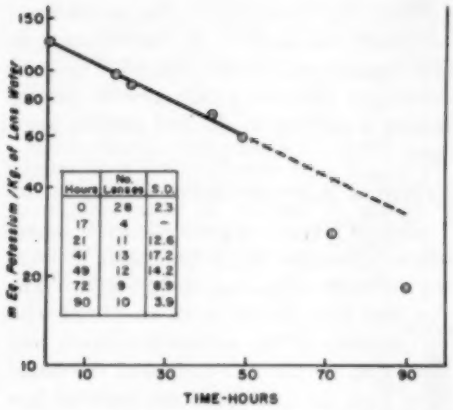

Fig. 2 (Harris, Hauschildt, and Nordquist). Potassium loss from the rabbit lens refrigerated at 0°C. in modified Tyrode's solution containing 200 mg.-percent glucose.

3 of the cation exchange which occurred when the extracted rabbit lens was refrigerated at 0°C. in a modified Tyrode's solution containing 200 mg. percent glucose. During the first 50 hours of refrigeration, the rate of loss of potassium was quite constant; thereafter, the loss was accelerated.

These observations are compatible with the view that the loss during the first 50 hours results simply from a block of the active transfer process. (For studies of the reversal of cation shift, refrigeration for any period up to 50 hours is thus justified.) The acceleration of the loss after 50 hours may result from degenerative changes.

On the other hand, a rapid gain in sodium occurred during the early hours of refrigeration. Thus, the initial gain in sodium probably reflects a migration of sodium chloride into the lens as well as an exchange of the cation with lenticular potassium. A similar rapid increase in chloride has been reported.[3] Lens sodium approaches a true equilibrium with the surrounding solution more quickly than does potassium. The net result is an increase in total base during refrigeration.

Scrutiny of the standard deviation indicates that the loss of potassium and gain of sodium during refrigeration may be quite variable from lens to lens. There are probably several reasons for this:

First, the lens is quite thick and variable diffusion into and from it would be anticipated. Uniform diffusion can be expected only in thin tissue slices.

Second, cutting the zonules and handling the lens must account to some extent for this variation since we have shown that the loss of potassium and gain of sodium is slowed if the undisturbed lens is refrigerated in the enucleated but intact eye.

Lastly, the exchange of cations during refrigeration varies somewhat with the substrate under test. Generally, those solutions which permitted the greatest cation exchange during refrigeration promoted recovery the least during subsequent incubation. This was not an invariable factor, however, and its significance cannot be stated at present.

Effect of glucose concentration on recovery of cation distribution

The effect of various concentrations of glucose on the ability of the refrigerated lens to regain its normal cation distribution was investigated. Figures 4 and 5 demonstrate that when glucose was absent from the media the lens failed to concentrate po-

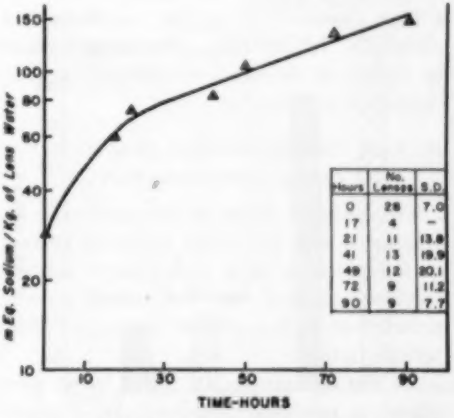

Fig. 3 (Harris, Hauschildt, and Nordquist). Sodium gain by the rabbit lens refrigerated at 0°C. in modified Tyrode's solution containing 200 mg. percent glucose.

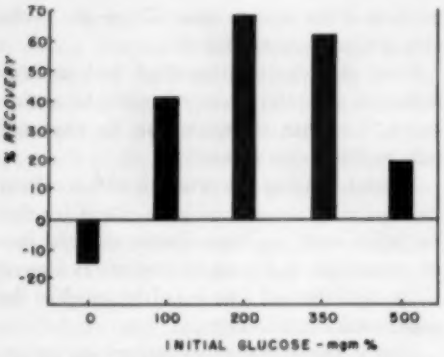

Fig. 4 (Harris, Hauschildt, and Nordquist). Effect of glucose concentration on reversal of the cold-induced potassium loss.

tassium and excrete sodium. When the initial glucose concentration was 100 mg. percent, a moderate recovery was found, that is, potassium re-entered and sodium was excreted from the lens in moderate degree. This concentration supplied glucose slightly in excess of the amount taken up by the lens during the period of observation.

When more adequate amounts of glucose were supplied by the media (200 to 350 mg. percent) the recovery reached a maximum. However, in high concentrations (500 mg. percent) the recovery declined markedly. It seems unlikely that this adverse effect of a high glucose level is due to diminished glycolysis. If anything, glucose utilization by tissues is increased as the glucose concentration is raised.[4]

Role of osmotic pressure in recovery of cation concentration

This reduced ability of the previously refrigerated lens to recover its cation content in the presence of a high glucose concentration suggested that the raised osmotic pressure of such a solution might be a deleterious factor.

To test this possibility, rabbit lenses were placed in solutions containing 200 mg. percent glucose and sufficient disaccharide (600 mg. percent) to produce an osmotic pressure equivalent to that of 500 mg. percent glucose. The recovery of the cation concentration was as great as that observed in 200 mg. percent glucose (fig. 6). Thus, the deleterious effect of a high glucose concentration is not due to elevated osmotic pressure.

Effect of ATP on cation recovery

Glucose enters the glycolytic cycle by reaction with adenosine triphosphate through the mediation of the enzyme hexokinase. The fact that high glucose levels interfered with the recovery of the sodium-potassium concentration by the lens suggested the possibility that the excess glucose depleted lenticular adenosine triphosphate to a harmful degree. (Adenosine triphosphate supplies the immediate energy for a variety of processes, including the phosphorylation of glucose and, perhaps, the active transfer of cations.)

Accordingly, adenosine triphosphate in a concentration of 0.001M was added to a modified Tyrode's solution containing 500 mg. percent glucose and its effect on the recovery of the cation distribution by the lens was determined. It was found to overcome completely the deleterious effect of the high glucose concentration (fig. 7).

Too much adenosine triphosphate proved toxic, however. Thus, the recovery of the cation concentration was impaired in a solu-

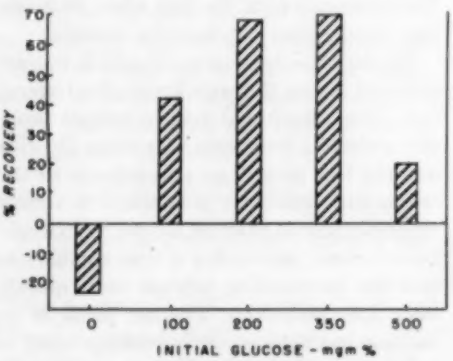

Fig. 5 (Harris, Hauschildt, and Nordquist). Effect of glucose concentration on reversal of cold-induced sodium gain.

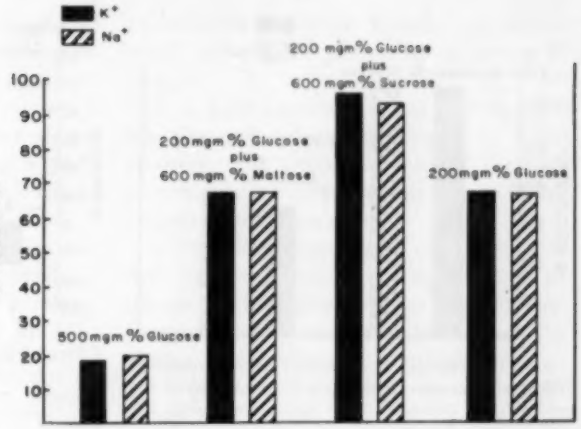

Fig. 6 (Harris, Hauschildt, and Nordquist). Effect on reversal of the cold-induced cation shift of disaccharides added in sufficient quantity to raise the osmotic pressure to the equivalent of 500 mg. percent glucose.

tion containing 0.002M adenosine triphosphate and 200 mg. percent glucose (fig. 8). These data suggest that the relative concentrations of adenosine triphosphate and glucose may be of significance. The efficiency of the cation reversal can be diminished by increasing the concentration of either.

The relationship of adenosine triphosphate and glucose focused attention on the hexokinase reaction and a possible effect of insulin. Among the suggested actions of insulin is the promotion of the phosphorylation of glucose to glucose-6-phosphate via the hexokinase reaction.[5]

It has been found that a direct tissue effect of insulin can be best demonstrated in tissues from diabetic animals. The molecular weight of insulin is reported to vary between 12,000 and 36,000 depending on the pH.[6] Thus, aqueous must contain very little insulin and one could raise a valid argument that the lens is normally a diabetic tissue in the sense that the hormone is denied access to it.

An in vitro effect of insulin on the lens might be anticipated. This concept was tested.

It was found that insulin in concentrations of 0.1 unit per ml. had no effect on the recovery of the cation concentration in modified Tyrode's solution containing 200 mg. percent glucose (fig. 9).

Ability of substances which enhance cation uptake to replace glucose as a substrate

During the course of our study, it has been observed that certain substances, notably l-glutamic acid and sucrose, enhance the cation reversal if adequate glucose is present. However, neither of these substances served as a substitute for glucose (fig. 10). Glucose or a similar source of energy is essential for cation transport. Results to be reported later indicate that other

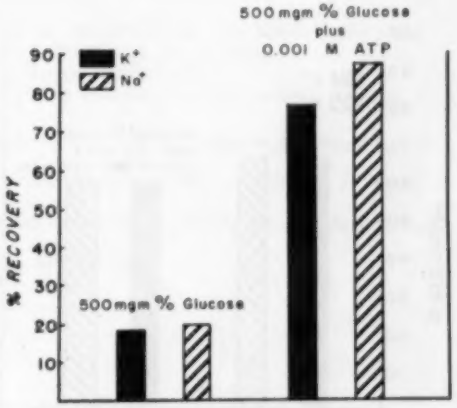

Fig. 7 (Harris, Hauschildt, and Nordquist). Effect of adenosine triphosphate on reversal of the cold-induced cation shift in media containing 500 mg. percent glucose.

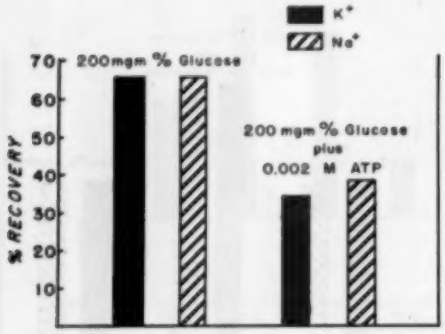

Fig. 8 (Harris, Hauschildt, and Nordquist). Effect of adenosine triphosphate on reversal of the cold-induced cation shift in media containing 200 mg. percent glucose.

compounds, such as fructose, may supply this energy.

Comment

As we have previously reported, when a lens loses potassium and gains sodium, it invariably becomes hydrated.[1] This was regularly observed in the present experiments, including those in which the lens was bathed in solutions with an elevated osmotic pressure. The hydration is reduced when the cation distribution returns to normal. The hydration of the lens, however, is influenced

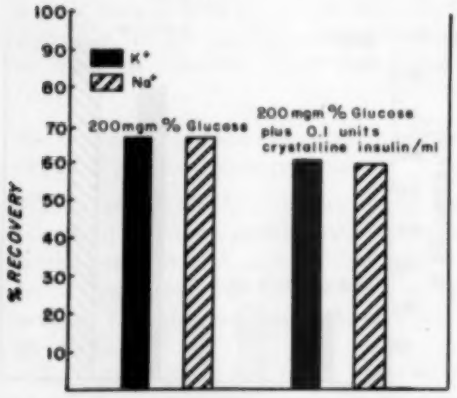

Fig. 9 (Harris, Hauschildt, and Nordquist). Effect of insulin on the reversal of the cold-induced cation shift of the lens. Results with insulin are not significantly different from results without it.

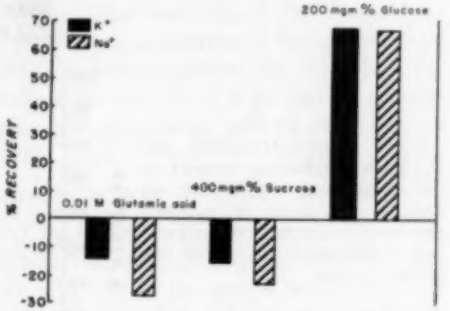

Fig. 10 (Harris, Hauschildt, and Nordquist). Effect of glutamic acid and sucrose on reversal of the cold-induced cation shift in the absence of glucose.

by other factors than the osmotic pressure differential as measured by the cation balance.[2]

The results affirm the concept that glucose is normally the major nutrient for the lens. Moreover, optimum concentration of this substance appears to be essential for proper lens function.

The thought frequently has been advanced that a high glucose level per se may be the factor responsible for the development of a diabetic cataract. This concept has received considerable support from studies of the alloxan-treated diabetic animal.[7]

Our results indicate that a high glucose level has indeed a deleterious effect on the lens. We have shown that this is not due to an increased osmotic pressure. Rather, some interference with the adenosine triphosphate available to the lens seems the likely cause since the effect of high glucose can be nullified if adenosine triphosphate is added. Measurements to date in our laboratory, however, have indicated that the high glucose level may not be the sole factor which brings about the shift in cations in the lens of an alloxan-treated diabetic animal. The problem requires further study.

It seems likely that conclusions to be drawn from these studies of the lens may apply to other tissues of the body. For example, when the diabetic patient goes into hyperglycemic coma, his cellular potassium

is depleted and cellular sodium increased.[8]

Part of the loss of cellular potassium is undoubtedly due to a loss of nitrogen and organic phosphate by the cell. However, a failure of the mechanism which controls the cation distribution must also play a part. Both an insulin deficiency and a high extracellular glucose level must contribute.

The studies here described support the clinical impression[9] that the treatment of diabetic coma should not include the administration of glucose solutions.

Summary

1. The role of glucose in the reversal of the cation shift induced in the lens by cold has been studied.

2. Glucose was found to be essential for the cation transport. Such substances as sucrose and l-glutamic acid which appears to enhance the active transfer of cations could not replace glucose as a source of energy.

3. A high concentration of glucose (500 mg. percent) reduced the degree of cation recovery. The untoward effect of the high glucose concentration was not due to an increased osmotic pressure.

4. The deleterious effect of a high glucose level was nullified by the addition of adenosine triphosphate. Added adenosine triphosphate was found to be toxic when the glucose concentration was normal.

5. Insulin had no effect on the cation transport of the lens.

6. The results are compatible with the view that a high glucose level per se may be the factor responsible for the development of a diabetic cataract.

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LENS METABOLISM AS STUDIED WITH THE REVERSIBLE CATION SHIFT*

II. The effect of oxygen and glutamic acid

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The present communication is concerned with the effect of oxygen and certain compounds metabolically related to glutamic acid on the reversal of the cation shift induced in the lens by cold. As has been noted,[1] such studies provide a measure of the role of various substances in the metabolism of the normal lens and may serve as a tool for the evaluation of the metabolic factors involved in the development and reversal of cataractous change.

Methods

The methods employed have been described in greater detail elsewhere.[1,2] Fresh rabbit lenses were placed in three ml. of a solution containing the substance under study, refrigerated at 0°C. for 41 to 45 hours, analyzed immediately, or incubated at 37°C. for an additional six hours. The basic solution was modified Tyrode's solution containing added bicarbonate and potassium and 200 mg. percent glucose. Other constituents were added as indicated. All amino acids used were the l-form. Where ionic substances were added, sodium chloride was deleted in sufficient quantity to maintain a constant osmotic pressure. Generally, the two lenses from the same animal were compared, one of the pair being analyzed after refrigeration, the other after refrigeration and subsequent incubation. A minimum of eight pair of lenses was used for any particular study. All results are expressed as percent recovery of the normal cation distributions.[3]

Effect of oxygen

The lens consumes a measurable but small amount of oxygen.[3] The enzymatic system which is involved in this oxygen consumption is not known with certainty. Indeed, it has been suggested that the oxygen consumption of the lens is entirely a nonenzymatic process.[4] Such a concept implies that the oxygen consumption produces no energy in a form that can be utilized by the lens and therefore has no role in the maintenance of normal lens function. The ability of the lens to concentrate cations provides an excellent test of this thesis.

The reversal of the cold-induced cation shift was tested in the basic medium with and without added glutamic acid, under aerobic and anaerobic conditions. It was found that when oxygen was excluded, the ability of the lens to recover its initial potassium and sodium concentrations was considerably reduced (fig. 1). The results leave no reasonable doubt that an oxygen supply is essential to the function of the normal lens.

An interesting speculation arises from

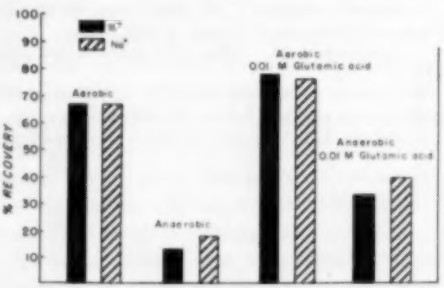

Fig. 1 (Harris, Hauschildt, and Nordquist). Comparison of effect of aerobic and anaerobic conditions on reversal of the cold-induced cation shift in the lens.

* From the John E. Weeks Memorial Laboratory, Department of Ophthalmology, University of Oregon Medical School. Supported by a grant provided by the U. S. Public Health Service.

these data. There is some evidence that the oxygen consumption of the lens is limited largely to the epithelium.[5] The epithelium contributes only a small fraction of the total quantity of potassium and sodium of the lens. Thus, the difference between the cation recovery under aerobic and anaerobic conditions cannot be accounted for by changes in the cation content of the epithelial cells alone. These results support the suggestion that the epithelium contributes energy for the entire lens.[6] They also are compatible with the hypothesis that an active transfer of cations occurs across the lens capsule.

Effect of glutamic acid

It has been shown that glutamic acid promotes the uptake of potassium and excretion of sodium by the previously refrigerated lens.[2] Similar results have been obtained with other tissues such as the brain and retina.[7] The exact mechanism of such action is not known. The present series of experiments have contributed the following information.

First, glutamic acid does not enhance the utilization of glucose or otherwise promote the action of this substance in cation recovery. Glucose uptake by the lens (as measured by disappearance from the solution) was not appreciably altered when glutamic acid was added to the media (table 1). (The decrease in rate of utilization with time is not unexpected.) It is known, too, that the amino acid cannot maintain cation recovery in the absence of glucose.[1]

TABLE 1

Effect of 0.01 M l-glutamic acid on glucose uptake by the rabbit lens

Period of Observation—(hours)	Glucose Uptake—mg./hr./lens	
	With Glutamic Acid	Without Glutamic Acid
6	0.24	0.26
11	0.22	0.22
16½	0.13	0.16
20½	0.17	0.16
24	0.13	0.16
Total Number of Lenses	13	15

Second, the beneficial effect of glutamic acid on cation recovery does not appear to be secondary to the production of energy by the oxidation of the amino acid via the tricarboxylic acid cycle. Thus, alpha-ketoglutaric acid, an intermediate in the oxidation of glutamic acid, failed to promote recovery of the cation content to the same degree as did the amino acid (fig. 2).

Third, the function of glutamic acid in cation recovery probably is not related to its capacity to bind ammonia produced by metabolizing cells. The amino acid takes up ammonia in certain tissues in the manner indicated in Figure 3. Intracellular ammonia may be toxic and the major metabolic function of glutamic acid in such tissues as the brain and retina may be the removal of ammonia.[8] When ammonia was added to the bathing solution the cation recovery was not altered significantly (fig. 4). Glutamine, however, was found to be toxic to the system under study (fig. 5). One might validly argue that the lack of toxicity of ammonia in the bathing medium does not rule out a possible toxic effect of ammonia more strategically placed. Free ammonia, however, passes cell barriers quite

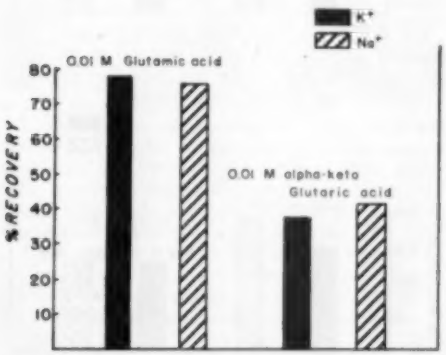

Fig. 2 (Harris, Hauschildt, and Nordquist). Comparison of the effect of a-ketoglutaric acid and glutamic acid on the reversal of the cold-induced cation shift.

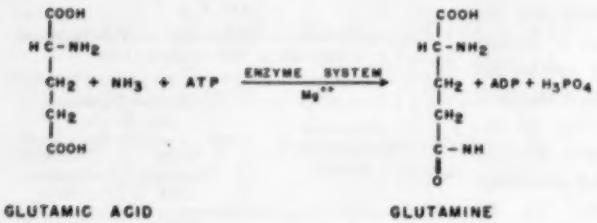

Fig. 3 (Harris, Hauschildt, and Nordquist). The metabolic relationship of glutamic acid to glutamine.

$$\text{GLUTAMINE} + H_2O \xrightarrow{\text{GLUTAMINASE}} \text{GLUTAMIC ACID} + NH_3$$

readily. Thus, the conclusion that this substance does not alter cation recovery is reasonable. The toxic effect of glutamine cannot be attributed to ammonia.

From the above data it can be concluded that the enhancing effect of glutamic acid on cation recovery is not due to any increased production of energy nor to the removal of ammonia. Davies and Krebs have suggested that glutamic acid plays a role in the transport system, the glutamic acid-iminoglutaric acid system serving as a carrier mechanism.[9] Our results with the lens, however, do not fully support this concept since we observed glutamic acid to be at least partially effective under anaerobic conditions (fig. 1).

Accepting for the moment the thesis that the steady-state of cations is a balance between their passive and active movements, the possibility that glutamic acid may simply

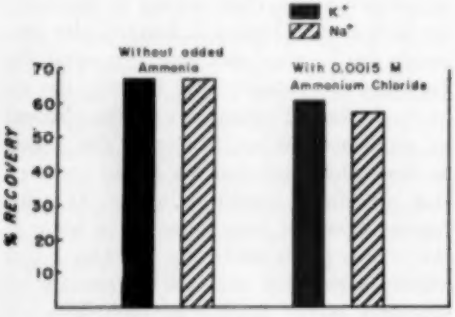

Fig. 4 (Harris, Hauschildt, and Nordquist). The effect of ammonia on reversal of the cold-induced cation shift. The difference is not statistically significant.

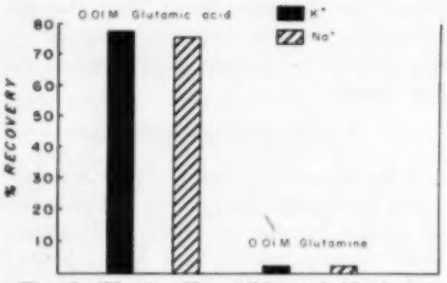

Fig. 5 (Harris, Hauschildt, and Nordquist). Comparison of the effect of glutamic acid and glutamine on reversal of the cold-induced cation shift in the lens.

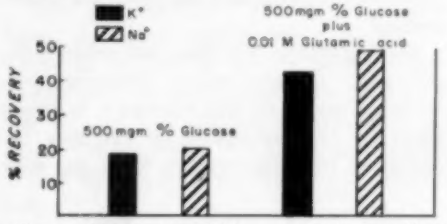

Fig. 6 (Harris, Hauschildt, and Nordquist). The effect of glutamic acid on the reversal of the cold-induced cation shift in the lens incubated in media containing high glucose concentration.

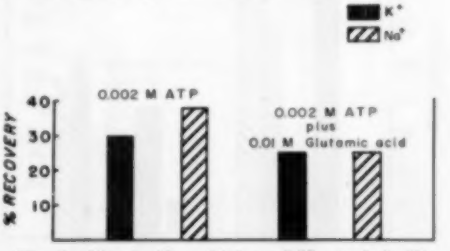

Fig. 7 (Harris, Hauschildt, and Nordquist). The effect of glutamic acid on reversal of the cold-induced cation shift in the lens incubated in media containing 0.002 M adenosine triphosphate.

reduce the passive movement must be credited. The ability of glutamic acid to overcome partially the deleterious effect of high glucose levels would support this view (fig. 6).

The amino acid is not as effective in this regard as adenosine triphosphate, which was observed to nullify completely the inhibitory effect of a high glucose level on cation recovery.[1] However, glutamic acid exerted no such protective action against a high concentration of adenosine triphosphate (fig. 7). We currently entertain the concept that the pyrophosphates are key substances in this delicately poised system, that their concentration and spatial distribution are both important, and that alterations of either may affect the active transport or the passive "leakage" rate. Glutamic acid and glutamine may conceivably alter the availability of the pyrophosphate.

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Discussion

Dr. Albert M. Potts (Cleveland): Dr. Harris' work is of particular interest to us in Cleveland because we are wrestling with a problem in the cornea similar to the one he is working with in the lens. Although there is a distinct lack of parallelism to date, the interest nevertheless exists.

I would like to ask Dr. Harris to expand on one part of his remarks, the function of the lens capsule metabolically. Ordinarily we think of these metabolic systems, which he diagrammed, as being connected with whole cell nuclei, and the cell nuclei in the lens of course are confined to the epithelium and the equator. I would like to know whether he has any evidence that the noncellular lens capsule has such an active metabolic function. Here again we are interested in possible corneal parallels.

Another question I would like to ask is whether he has tried any of the other cataractogenic monosaccharides. The sugars which he attempted to substitute for glucose were disaccharides or monosaccharides that are known to be metabolized in the diabetic individual. Galactose in particular would be a sugar of interest to try in this particular case.

The third question is whether the postulated effect of glucose on the system by using up adenosine triphosphate, which is a very interesting explanation for the effect of adenosine triphosphate in reversing the glucose inhibition, is paralleled in ordinary glycolytic schemes. It was my impression that the hexokinase system was generally considered to be limiting in glycolysis, and that its concentration determined the rate at which glycolysis would go, and it was difficult by adding extra glucose to use up any of the other metabolites.

Couldn't it be possible that glucose might inhibit the re-synthesis of adenosine triphosphate farther down the line?

Dr. P. J. Leinfelder (Iowa City, Iowa): I would like to ask Dr. Harris how he maintained the pH of his solutions when his experiments ran over periods of 24 and 48 hours and longer.

Dr. John E. Harris (Portland, Oregon): In answer to Dr. Potts' questions, we have studied the question of the lens capsule, and feel that evidence

definitely favors the view that active transport occurs across it, or at least that a limiting barrier is immediately adjacent to it. This evidence comes from many experiments. First, if one simply nicks the lens capsule, an irreversible loss of potassium and gain of sodium results. I know of no better way to explain this effect than to consider that some active transport of cations occurs across the lens capsule.

Secondly, the decrease in the cation recoverability which results when oxygen is excluded from the medium is much greater than would be predicted if one considered that the difference represents a cation exchange of only those cells which utilize oxygen. You will recall that the oxygen consumption of the lens is probably confined to the epithelium and a small amount of peripheral cortex. Our results support the view that the necessary energy is supplied by these cells and delivered for use elsewhere in the lens, quite likely, to the lens capsule.

Third, the effect of adenosine triphosphate on this particular system is most likely exerted at the lens surface since it seems improbable that this molecule diffuses into the lens to any considerable degree. Again, this points to activity at or near the lens capsule.

We have tried other monosaccharides on our particular system and these results will be reported in another paper. The reason we used a disaccharide to rule out an osmotic effect was because we wanted to stay away from substances such as xylose and galactose which might conceivably affect our system. Galactose does not provide energy for the active transport system at all. I can say at the moment that our observations are compatible with those of Dr. Patterson of Cleveland on the development of cataract in diabetic animals fed excess galactose. This is quite striking considering the diverse experimental approach.

As far as the inhibition of the adenosine triphosphate synthesis by a high glucose level is concerned, I see no reason to believe that such would be the case. We consider the effect of excess glucose to be not one of depletion but one of a binding. The adenosine triphosphate is prevented from exerting its normal action at some critical point, possibly at the lens capsule. One must assume that the glucose breakdown follows the usual cycle and that the rate of utilization of glucose and the production of adenosine triphosphate is not lowered. Wick, as you recall, studied the effect of the glucose level on the rate of glucose utilization and found that even in the diabetic animal utilization was enhanced at high glucose levels. There are no comparable experiments on the lens but I would anticipate the same effect.

As far as the effect of co-enzyme I is concerned, Dr. Dische, we found it did not appreciably alter cation recovery. We have not tried co-enzyme II, but I think it would be an excellent idea. We have done multiple experiments on the effect of glutathione and cysteine with the thought that our observed effect of glutamic acid might be tied in with glutathione, particularly since Kinsey and his colleagues, using marked glycine, showed that glutathione undergoes constant turnover. Our evidence so far does not indicate that the effect of glutamic acid is exerted in this manner. I don't feel our experiments are conclusive, however, and thus we have not reported them as yet.

In regard to the pH, Dr. Leinfelder, our medium is a modified Tyrode's solution containing considerably more bicarbonate than the initial formula specified. It is overlaid with a mixture of carbon dioxide and oxygen or carbon dioxide and nitrogen. The final pH lies between 7.32 and 7.48. The change in pH on refrigeration is slight. The alteration during subsequent six-hour incubation, however, is a little bit toward the acid side, down to 7.2. We have run control studies and have found no effect of that pH change in the range experimentally observed.

EFFECTS OF INTRACAROTID INJECTION OF A BASIC DYE ON THE CILIARY BODY*

ELMER J. BALLINTINE, M.D., AND LAWRENCE PETERS, M.D.
Cleveland, Ohio

I. INTRODUCTION

Intravenous administration of a number of basic dyes (including some cyanine and styrylquinoline dyes) is followed by their rapid accumulation in the tubular epithelium of the kidney in high concentration. Appropriate doses produce marked renal functional impairment accompanied by relatively mild histologic changes confined largely to the cells of the proximal convoluted segment where dye concentration is highest.

In the elucidation of the biochemical basis of this nephrotoxicity of a series of styrylquinoline and cyanine dyes, one of us (Peters[1]) has demonstrated that some of the dyes have profound effects on certain metabolic processes. These effects, summarized later herein, suggested that the dyes might be useful in investigating metabolic processes related to aqueous-humor secretion.

II. SUMMARY OF METABOLIC PROPERTIES OF THE NEPHROTOXIC DYES

A. CHEMICAL PROPERTIES

A study of 13 dyes showed that a basic quaternary resonating structure was necessary for activity. The structural formulas of the two dyes selected for this study (*N* — (2-(p-Diethylaminostyryl) — 6 — methylquinoline) ethochloride, No. 350 and *1′* — Ethyl — 3,6, — dimethyl — *2* — phenyl — 4 — pyrimido — *2′* — cyanine chloride, No. 863, Figure 1—a and b) show these essential structural features. In each, there are two alkyl or aryl substituted nitrogen atoms, one tertiary and one quaternary, connected by a chain of carbon atoms with alternate double and single bonds. The structure can also be written with the positions of the nitrogen atoms and the double and single bonds interchanged (fig. 1-c). The exact positions of the double bonds and the positive charge of the nitrogen atom are indeterminate, and the dyes therefore exhibit resonance.

These dyes are concentrated in the cells of renal tubules and are visible in the epithelium of the ciliary body and other epithelial tissues after intravenous injection. This concentration is demonstrable in experiments performed in vitro where it is not prevented by anaerobiosis.

Dyes No. 863 and No. 350 were selected for this study, the former because considerable information concerning its pharmacologic properties was available from other

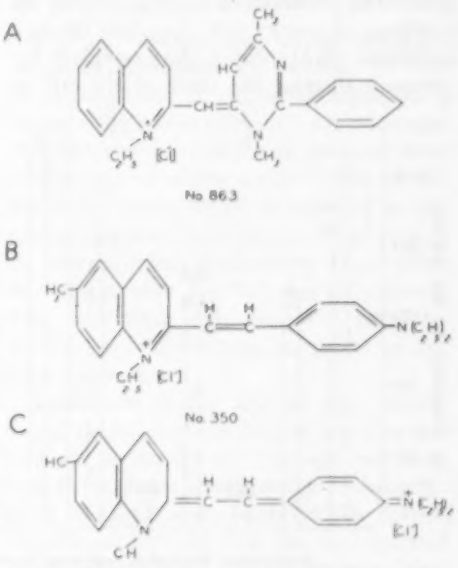

Fig. 1 (Ballintine and Peters). Structural formulas of the dyes selected. (See text.)

* From the Western Reserve University School of Medicine and The University Hospitals of Cleveland. This study was supported in part by research grant B 121 (C) from The National Institute of Neurological Diseases and Blindness of the National Institute of Health, Public Health Service.

studies in this laboratory; and the latter because of its potency in low concentrations in inhibiting respiration in mitochondrial systems.

B. Effects on oxidative metabolism

Dye No. 863 depressed the oxygen uptake of a cell-free homogenate of rabbit kidney cortex during oxidation of endogenous substrate or added lactate or acetate. For example, the O_2 uptake of this homogenate with added lactate was reduced to 35 percent of control values on addition of 1×10^{-4} molar No. 863. This inhibition was not reversed by addition of a large excess of diphosphopyridine-nucleotide (5×10^{-3} M stabilized with 1×10^{-2} M nicotinamide).

Dyes No. 350 and No. 863 inhibited the oxygen consumption of mitochondrial preparations from rat liver and from rabbit kidney, the so-called "cyclophorase" preparation of Green[2] with added succinate as a substrate. This system is capable of oxidizing succinate, α-ketoglutarate, and pyruvate completely by way of the tri carboxylic acid cycle. The oxidation is accompanied by the synthesis of energy rich phosphate (that is, adenosine triphosphate). An exemplary experiment showing the effect of No. 863 on the oxidation of succinate in this system and its reversal by cresyl blue is summarized in the graph (fig. 2).

Cresyl blue is an artificial electron carrier which bypasses the cytochrome system in transferring electrons to molecular oxygen. The reversal of inhibition indicates that the inhibitor is acting at the cytochrome-cytochrome oxidase stage of oxidation by molecular oxygen.

Bueding,[3] in this laboratory, had shown previous to these studies that dye No. 863 inhibited cytochrome oxidase, but did not affect cytochrome in a system involving only these two substances and a synthetic substrate.

C. Effects on oxidative phosphorylation

In a "cyclophorase" preparation containing succinate or α-keto glutarate as a substrate and with hexokinase and glucose added to trap any organic phosphate formed as glucose-6-phosphate, concentrations of No. 863 (0.33×10^{-3} M) which produce only a mild inhibition of oxygen uptake at 25°C. produced almost complete inhibition of the concomitant aerobic synthesis of organic (for example, "energy rich") phosphate. This dissociation, or uncoupling of phosphorylation from oxidation, was marked in

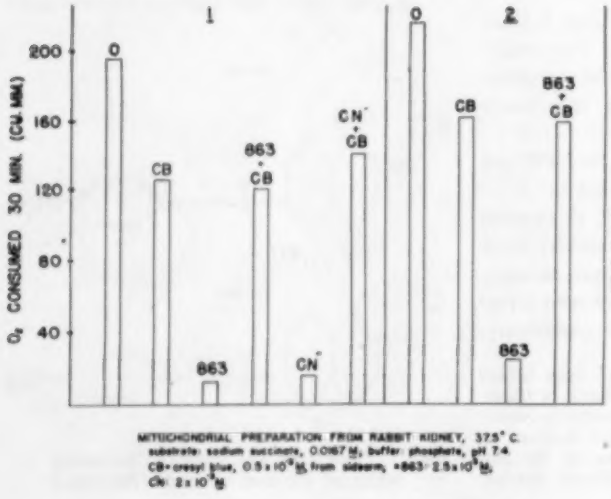

Fig. 2 (Ballintine and Peters). Effect of dye No. 863 on oxidation of succinate in the cyclophorase system and its reversal with cresyl blue.

TABLE 1

INHIBITION OF OXIDATIVE PHOSPHORYLATION BY DYE NO. 350

(Method used was that of Cross and others.[4] Observations made at 25°C. Substrate 0.01 M α Ketoglutarate.)

Inhibitor Concentration $\times 10^{-3}$	O_2 Uptake μ Atoms	Inorganic P Disappearance μ Atoms	P:O Ratio
0	8.3	17.4	2.10
0	8.0	19.1	2.39
0.01	3.7	7.0	2.28
0.033	2.7	1.0	0.37
0.10	2.6	1.3	0.50
0.33	2.6	0.33	0.13

the case of No. 863 and less marked with No. 350 where the effect on oxidation was much more pronounced.

An exemplary experiment with α-ketoglutarate (0.01 M) as a substrate and dye No. 350 as the inhibitor is shown in Table 1. The method used was that of Cross, and others.[4] The observations were made at 25°C. because of the instability of phosphorylating enzymes of cyclophorase at 37.5°C.

The accompanying graphs (figs. 3 and 4) summarize the data obtained from such studies, each point on the graph being the average of results of several measurements at each dye concentration. Abscissae are dye concentrations, ordinates are activities expressed as percentage of simultaneously observed controls. The graphs show that in addition to partially inhibiting oxygen uptake, the dyes in lower concentration have a more marked inhibitory effect on phosphorylation. The well known effect of 2,4-dinitrophenol in dissociating oxidation from phosphorylation in this preparation is shown for comparison (fig. 5).

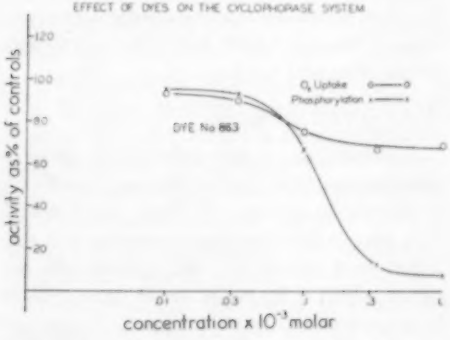

Fig. 3 (Ballintine and Peters). Effect of dyes on the cyclophorase system.

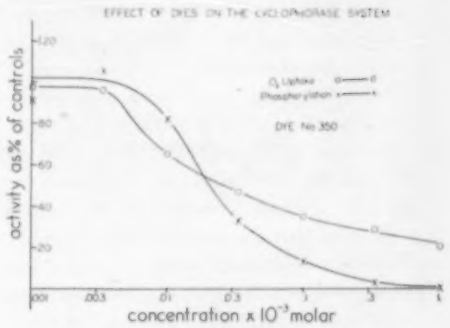

Fig. 4 (Ballintine and Peters). Effect of dyes on cyclophorase system.

III. PROCEDURES FOR ASSESSING EFFECTS OF DYES ON THE CILIARY BODY-AQUEOUS HUMOR SYSTEM IN VIVO

A. CHOICE OF DYES FOR STUDY IN VIVO

Because these dyes were strongly absorbed in ciliary epithelium and because of their effect on cellular oxidation when present in high enough concentration, it was postulated that under proper conditions, administration of the dyes to albino rabbits might be followed by inhibition of respiration of the ciliary processes without significant effects on other tissues. Preliminary experiments were begun with No. 863, but experiments were continued with No. 350 when its greater inhibitory action on oxidation became apparent.

Subsequent to the first in vivo experiments, the effect of the dyes on phosphorylation was demonstrated. Although this effect would complicate interpretation, it was decided to continue the study of No. 350 in rabbit eyes because this dye had marked effects on oxidation at concentrations that permitted appreciable oxidative phosphory-

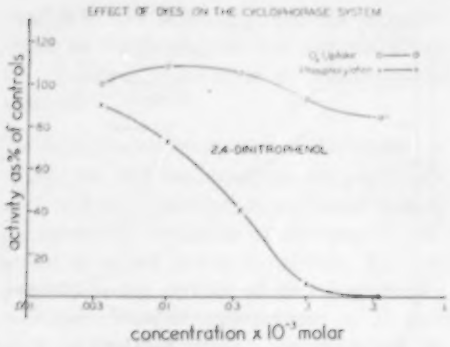

Fig. 5 (Ballintine and Peters). Effect of dyes on cyclophorase system.

lation, and because most theories postulate no need for energy rich phosphate in secretion of aqueous humor while the integrity of the epithelial cytochrome oxidase is considered to be essential.

It should be noted, however, that renal cortical slices made from kidneys of rabbits (and dogs) given a nephrotoxic dose of dye No. 863 a few hours earlier demonstrated no impairment of oxygen uptake.[8] Despite this, cyclophorase prepared from the cortices of such kidneys demonstrated a markedly reduced rate of oxidative phosphorylation when compared to uninjected controls. Slices made from kidneys removed 24 to 48 hours after dye injection did demonstrate a decrease in oxygen uptake. This delayed effect on oxidation in the slice is probably secondary to cellular injury following prolonged impairment of oxidative phosphorylation for the 48 to 96 hours that the dye persists in the kidney.

B. Measurement of oxygen uptake of the ciliary processes of the albino rabbits

Rabbits were killed by decapitation, and the eyes enucleated and placed in cold Krebs-Ringer phosphate solution (pH 7.35). In a cold laboratory (3° to 5°C.) the eyes were bisected at the equator and lens and vitreous swept off the anterior half.

The cornea and attached rim of sclera were everted and spread on a jig so that the ciliary processes attached to the posterior surface of the iris were exposed. The iris was divided into symmetric halves by two radial cuts. Each half iris with attached ciliary processes was stripped from the underlying tissues and placed in the main chamber of a Warburg vessel (5 ml. cap.) containing 0.8 or 1.0 ml. of 0.01 M succinate in Krebs-Ringer solution (pH 7.35). Alkali (0.1 ml. 6 *N* NaOH) was placed in each center well.

Respiration was measured for one hour in the Warburg bath at 37.5°C. after gassing with oxygen and equilibrating. Oxygen uptake was expressed as cu. mm. per eye, that is, as the sum of the values for the two vessels from each eye, after application of constants. Respiration was increased 50 percent or more by the presence of succinate. Pyruvate caused no significant increase. Utilization of succinate was not increased by lowering pH as in some tissues more readily penetrated by the monosodium salt. Oxygen uptake ranged from 50 to 80 cu. mm. in different rabbits whose weight varied from 2.5 to 3.5 kg. with a concomitant variation in size of eyes.

When ciliary processes were dissected free of the supporting iris, respiration of the two components was approximately equal, but the sum of the respirations for the two components was less than that of the intact combination, because of injury during dissection. Hence, the combined iris-ciliary processes preparation was used.

C. Analytic methods

Aqueous humor was collected by anterior chamber puncture with a 26 gauge needle on a tuberculin syringe. In those cases where CO_2 was to be determined aqueous was collected in a special 0.2 ml. pipette into the tip of which a 26-gauge needle was secured by a ground and cemented joint. To prevent loss of CO_2 after collection, the needle was

sealed by sticking it into a rubber block and only the fluid contained in the mid portion of the long capillary pipette was used for CO_2 determination. Heart blood was collected in a 10 cc. heparinized syringe and the samples for CO_2 determination were stored under oil.

Ascorbic acid was determined colorimetrically by a modification of the method of Lowry, Lopez, and Bessy[6] using 0.06 to 0.015 ml. samples for aqueous and 1.0 or 0.5 ml. samples for blood.

Total CO_2 content of blood and aqueous was determined by the method of Van Slyke[7, 8] for 0.2 ml. samples, using 0.1 ml. samples.

D. Intraocular pressure

Intraocular pressure was measured with a Schiøtz tonometer carrying the 5.5-gm. weight. Tonometer readings only were recorded since no conversion table for rabbit eyes was available.

E. Method for production of high concentration of dye in ciliary epithelium in vivo

1. Steps in development of a method for production of high concentration of cyanine and styrylquinoline dye in the ciliary epithelium in vivo:

a. Intravenous injection of No. 863 into albino rabbits even in nephrotoxic doses resulted in only moderate staining of the ciliary epithelium in both eyes. The respiration of the ciliary body was not depressed when measured one hour after injection.

b. In order to present higher concentrations of the dye to the ciliary epitheluim, injections into the common carotid artery were attempted. Local anesthesia was used in early experiments but inability to immobilize animals completely permitted sufficient struggling to interrupt injection.

Intracarotid injection under pentobarbital anesthesia in four rabbits resulted in more marked staining of the ciliary epithelium than did systemic intravenous injection, and staining was confined to the ipsilateral eye. Respiration of the ciliary body did not differ significantly from the contralateral control.

In three additional rabbits, a solution of No. 863 (4.0 to 6.0 mg./kg.) was injected into the common carotid on one side after ligation of the external branch. An equal volume of solvent was injected into the opposite carotid under identical circumstances. Intense staining of the ciliary epithelium resulted in the eye on the injected side. In two of three cases the respiration of iris and attached ciliary body, removed one to one and one-half hours after injection, was significantly depressed as compared to the contralateral control.

c. Attention was shifted to dye No. 350 when its greater inhibitory action on oxidative metabolism became apparent.

In 15 rabbits successfully injected, after ligation of the external carotid, with doses of 2.0 to 6.0 mg. of dye per kg., eyes removed from the injected side one to one and one-half hours after injection revealed intense staining of the ciliary body, confined to the epithelium, with no staining of the underlying iris. The red color of No. 350 permitted more accurate appraisal of the degree of staining than did the yellow color of No. 863.

Staining of the retina also occurred, as did less intense staining of the equatorial zone of the lens. Respiration of the ciliary body was definitely depressed in 11 instances. Intraocular pressure, measured before decapitation, and the ascorbic acid level of the aqueous humor on the injected side, also were depressed in some instances.

d. Twenty additional animals were subjected to the same injection procedure with the modification that the neck incision was closed with sutures so that the animal could be held for observation. The mortality rate was high in this group. Some animals died within a few hours after injection because of respiratory obstruction. Various forms of

anesthesia were used in attempting to circumvent this difficulty. Open-drop ether was most conducive to survival.

Delayed death (48 to 120 hr.) was also caused by respiratory obstruction which, however, did not develop until after a period of normal behavior. This was attributed to edema of the tongue, larynx, and pharynx, caused by ischemia resulting from insufficient collateral circulation for the bilaterally ligated external carotids. When the injections were made during temporary occlusion of the external carotid with a special noncrushing clamp, ischemic edema did not occur.

2. Standard technique for intracarotid injection:

As a result of the above experiments the following technique was adopted. Under light ether anesthesia a loose ligature was passed beneath each common carotid, isolated through a longitudinal neck incision. The bifurcation of one carotid was isolated and the external carotid was occluded with the noncrushing clamp. The common carotid was steadied with the ligature while a 26-gauge needle was inserted into the lumen.

The ligature was relaxed and 3.7 ml. of a solution of dye No. 350 (1.25 mg. per ml.) in five-percent glucose was injected over a period of one and one-half to two minutes. The clamp was released, the needle withdrawn, and a pad of oxycel applied to the puncture site before removal of the ligature. Injection of solvent only into the other carotid was carried out by the same procedure. The neck was closed with silk sutures.

The red color of the dye could be seen grossly through the albino iris on the injected side. It was still visible in the exposed ciliary processes in animals killed at 24 hours, but not later. Microscopic examination showed the dye concentrated in the epithelium of the processes.

Despite the earlier solution of several problems relating to mortality, there still were rabbits which did not survive this finally adopted procedure. The neck dissection produced some edema with slight tracheal compression and laryngospasm during the first 12 hours after operation. In otherwise healthy rabbits this was not a serious complication, for example, of the first 11 and the last 10 consecutive operations four were fatal and the others were well tolerated. On the other hand, an intermediate group was characterized by a high incidence of upper respiratory infection, apparently epidemic in the supplier's colony. This resulted in an overall mortality of 35 percent in the 94 rabbits subjected to this procedure.

In five rabbits no visible staining of the eye occurred. This was attributed to either faulty injection or anomalies of circulation. Of the 61 survivors, 12 were used for anatomic and histologic studies, 30 were used for metabolic studies reported here, and 14 were used for other purposes such as prolonged observation, and other metabolic studies.

The intraocular pressure was measured and recorded periodically. After various selected survival periods, up to four weeks, ascorbic acid (35 mg./kg.) was injected intraperitoneally. One hour later specimens of heart blood and aqueous humor were collected for ascorbic-acid analysis.

The ascorbic acid was given because the effect of the dye might be merely to reduce the volume of aqueous secreted without a change in composition. If so, the measurement of aqueous ascorbic while the aqueous level was rising, and before the aqueous humor had reached a new steady state, might reveal a defective secretory process in one eye. In addition the large amount of ascorbic being offered to the secretory mechanism might saturate the impaired capacity of one eye. After collection of specimens, the irises with attached ciliary processes were submitted to respirometric studies.

In two exemplary experiments with injection of dye into the right carotid, the results shown in Table 2 were obtained:

TABLE 2

RESULTS IN TWO EXEMPLARY EXPERIMENTS WITH INJECTION OF DYE INTO THE RIGHT CAROTID

RABBIT A					
1. *Intraocular pressure*					
Time after injection (hr.):		9	19	48	
Tonometer reading	O.D.	9	8	6	
	O.S.	3	4	3	
2. *Terminal studies at 48 hours*					
(a) O_2 uptake		*O.D.*		*O.S.*	
(cu. mm./hr.)		21		40	
		26		39	
		—		—	
Total		47		79	
(b) Ascorbic acid in aqueous humor (γ/ml.)		386		435	
RABBIT B					
1. *Intraocular pressure*					
Time after injection (days):		3	11	19	21
Tonometer reading	O.D.	11	9	6	6
	O.S.	5	3	5	5.5
2. *Terminal studies at 21 days*					
(a) O_2 uptake		*O.D.*		*O.S.*	
(cu. mm./hr.)		24		26	
		25		27	
		—		—	
Total		49		53	
(b) Ascorbic acid in aqueous humor (γ/ml.)		146		306	

IV. RESULTS

A. ANATOMIC CHANGES

1. Gross effects at injection site:

Two rabbits which had been subjected to the injection procedure three and one-half and five and one-half months previously, respectively, were anesthetized and the neck dissections were repeated. There was minimal scarring in the operated regions, the oxycel pads had completely disappeared, and the carotids and their branches were easily isolated and found to be of normal diameter and appearance with normal arterial pulsations.

2. Histologic changes:

Ten albino rabbits were subjected to the injection technique described and killed at intervals. In all animals, the injection produced visible staining of the eye and prolonged lowering of the intraocular pressure on the side receiving dye. Histologic sections of the eyes from the sides receiving dye were compared with sections from control eyes.

The most obvious early changes in the treated eyes from rabbits sacrificed 48 hours after injection were marked hyperemia with vascular dilatation and edema of the ciliary processes. The edema involved the entire structure of the process. The epithelium of the process was intact. These changes were also found to a lesser degree in several of the treated eyes from animals sacrificed at longer intervals.

In the retina the most common change found was vacuolization of the nerve-fiber layer. Additional later changes found in some areas were loss of the internal nuclear layer, degenerative changes leading to granularity of the rod layer, loss of the external nuclear layer, and finally nearly complete disorganization and destruction of the retina with adhesions between the choroid and retina. In every treated eye some of these changes were present. No inflammatory cellular infiltration was found in any of the eyes examined.

B. EFFECTS ON INTRAOCULAR PRESSURE

Of 31 rabbits subjected to the same procedure already described, except for variation in period of observation, intraocular pressure was depressed in 25 rabbits at the

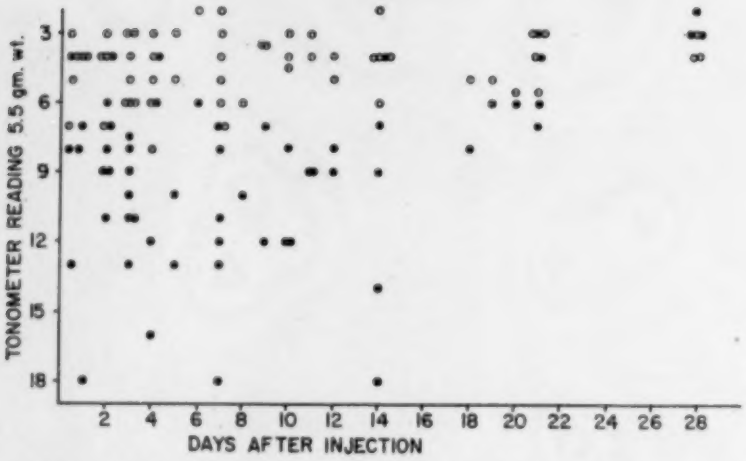

Fig. 6 (Ballintine and Peters). Effect of dyes on intraocular pressure. (Solid circles = treated eyes; open circles = control eyes.)

time of death. In all five rabbits killed at three weeks or beyond, the intraocular pressure on the injected side had returned to normal after being depressed at all intervals up to two weeks. The graph (fig. 6) shows the range of tonometer reading obtained. There are many more pairs of readings than rabbits because serial measurements were made on most animals.

C. Effects on oxygen uptake

The oxygen uptake of iris-ciliary body preparations from 29 rabbits subjected to the dye injection procedure was measured by the technique described. Oxygen uptake of the preparation from the eye receiving the dye was reduced by more than 20 percent of that of the control eye in nine of 16 rabbits sacrificed not later than 96 hours after injection. In 13 rabbits killed at intervals beyond 96 hours, oxygen uptake was reduced in only 3. In only one rabbit, (killed four weeks after injection) was the oxygen uptake of the injected side higher than that of the control (fig. 7).

D. Ascorbic acid in aqueous humor

The concentration of ascorbic acid in the aqueous humor from untreated eyes ranged from 205 to 670 γ per ml. The plasma range was 8.5 to 133. γ per ml. The concentration of aqueous-humor ascorbic acid from the injected side was reduced to 70 percent or less of the control value in 17 of 28 rabbits. In those killed at 12 hours or less there was a reduction in two of five; in those killed at 48 hours there was a reduction in four of seven; and in those killed beyond 48 hours there was a reduction in 11 of 16. In one rabbit killed at one hour and one at four weeks there was an insignificant increase in the treated eye. In one rabbit killed at one hour the level in the treated eye was definitely higher. The graph (fig. 8) shows the reduction in the treated eye.

In the determination of aqueous ascorbic acid, when the specimen of aqueous was added to the metaphosphoric acid solution, in most cases the precipitate of protein was minimal or absent. In a few cases, especially those analyzed in the first 12 hours after operation, there was an appreciable protein cloud produced but in even the most extreme cases, the density of precipitate was minimal when compared to that produced by plasma.

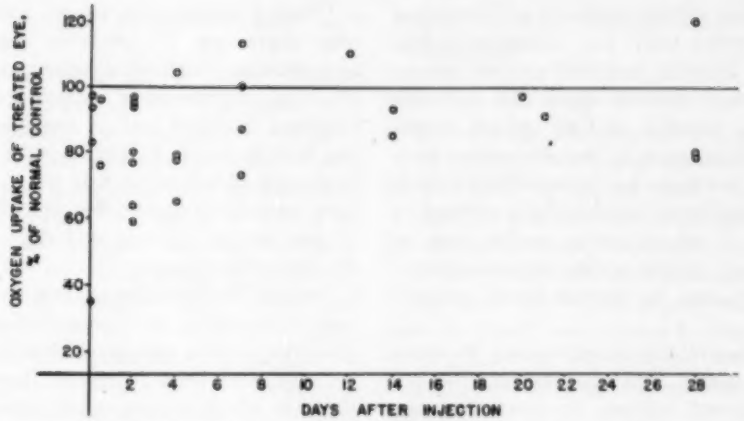

Fig. 7 (Ballintine and Peters). Effect of dyes on oxygen uptake.

E. Total CO_2 in aqueous humor

Total CO_2 in aqueous humor from seven rabbits subjected to the injection procedure was determined. In one rabbit killed at one hour no reduction in CO_2 was found. In three killed at 48 hours, one at 96 hours, one at seven days, and one at 12 days, the CO_2 in the aqueous from the treated side was reduced from six to 39-percent of that from the control eye. In all cases except one, the CO_2 of the aqueous from both eyes was higher than the CO_2 of simultaneously sampled whole blood. The one exception was in the case where the reduction was 39 percent.

These observations are too few to permit drawing conclusions and additional animals are being prepared for CO_2 determination.

F. Correlations

The function most consistently impaired by the presence of the dye was the intraocular pressure. Because this was, of necessity, measured by an arbitrary, and non linear scale, detection of correlation between the degree of intraocular pressure depression and other functions is difficult.

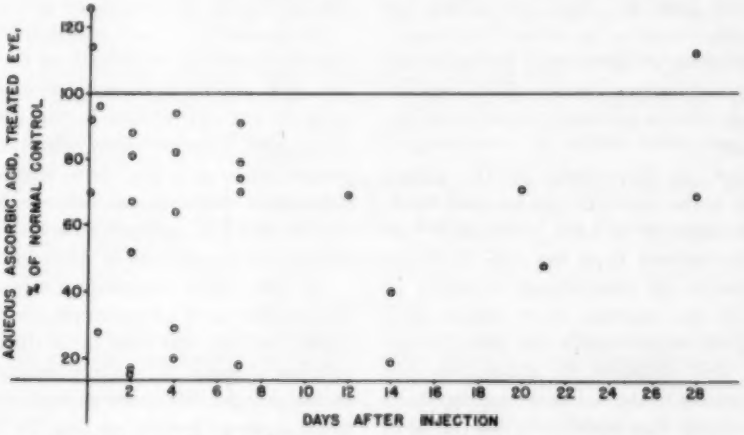

Fig. 8 (Ballintine and Peters). Effect of dyes on ascorbic-acid content of aqueous humor.

Of those rabbits sacrificed at 96 hours or less, in seven there was reduction of both aqueous ascorbic acid and oxygen uptake. In five there was no appreciable reduction of either ascorbic acid or oxygen uptake despite depression of the intraocular pressure. In two there was appreciable reduction of aqueous-humor ascorbic acid without reduction of oxygen uptake and in two, reduction of oxygen uptake without appreciable reduction in aqueous-humor ascorbic acid.

In those rabbits killed beyond 96 hours, oxygen uptake and aqueous ascorbic acid were reduced in three. In seven there was reduction of ascorbic acid level but no reduction of oxygen uptake. In two there was no reduction of either oxygen uptake or ascorbic acid. In two killed at four weeks there was depression of oxygen uptake but the intraocular pressure was normal.

V. Discussion and Conclusions

The absence of clear-cut correlations among the observations makes unequivocal interpretation difficult and a complete explanation of the effects observed must await further investigation. However, the original inference that these dyes offered a means of influencing the metabolism of the ciliary body by concentration in ciliary epithelium has been substantiated to the extent that consistent depression of intraocular pressure was produced in the treated eyes without destructive changes in the histologic structure of the ciliary body.

Whether the hyperemia of the ciliary processes is the result of the lowered intraocular pressure or of some other cause cannot be determined from the data available. The presence of insignificant amounts of protein in the aqueous from treated eyes having a depressed intraocular pressure and ascorbic acid supports the conclusion that the depression is the result of metabolic inhibition rather than nonspecific alteration of permeability.

Detailed examination of the data shows that depression of oxidative metabolism alone does not explain the sequence of events observed. Depression of oxygen uptake was observed in about half of the eyes within the first 96 hours. Oxygen uptake was not depressed in the other half of the treated eyes measured during the first 96 hours despite definite staining and depression of the intraocular pressure.

Beyond 96 hours the oxygen uptake was normal in most of the treated eyes indicating recovery in those eyes in which it might presumably have been depressed. However, in those in which oxygen uptake was normal, aqueous ascorbic acid was reduced and the intraocular pressure remained depressed until some time between two and three weeks after injection. In those animals killed at three weeks and beyond, the intraocular pressure had returned to normal but the ascorbic acid was depressed in three of five.

The demonstration that dye No. 350 inhibits oxidative phosphorylation at concentrations lower than those inhibiting oxidation suggests that depression of the intraocular pressure might be the result of inhibition of phosphorylation (or some other effect) and that depression of oxidation might be only an incidental finding in eyes which happened to absorb the highest concentrations of dye in ciliary epithelium.

The recovery of each function at a different time could be explained on the assumption that inhibition of some system or systems by the dye results in the destruction, exhaustion, or irreversible inhibition of substances necessary for maintenance of the intraocular pressure, ascorbic-acid concentration, and CO_2 concentration and that these substances regenerate at different rates.

An alternative explanation might be that the inhibition of phosphorylation results in cellular injury, too mild to produce obvious cellular changes yet severe enough to increase nonspecifically the permeability of the blood aqueous barrier so that the reduction of the intraocular pressure and aqueous

ascorbic acid and CO_2 are the result of back diffusion.

There may be an analogy to the effects of the dye on kidney where a nephrotoxic dose of dye No. 863 can produce impairment of phosphorylation without impairment of oxygen uptake. Later, impaired oxidation develops, apparently the result of cellular injury, which produces minimal histologic changes.[5] If the effects observed in eyes are the result of "cellular injury" or increased permeability then the recovery of the intraocular pressure before the return of aqueous ascorbic acid requires recovery of impermeability to different substances at different times.

Additional information, especially with respect to histologic changes and the concentration of ascorbic acid and CO_2 content of the aqueous humor from the eyes receiving dye No. 350 is being obtained by increasing the number of animals in the experimental series. It is planned to attempt to investigate further the role of phosphorylation in aqueous humor formation using dyes such as No. 863 which inhibit oxidation phosphorylation under conditions and in concentrations which are only slightly inhibitory to oxidation per se.

VI. Summary

1. Intracarotid injection of styrylquinoline dye No. 350 in the albino rabbit resulted in marked staining of the ciliary epithelium without destructive morphologic alteration or cellular inflammatory infiltration.

2. The intraocular pressure and the ascorbic acid and CO_2 concentrations in the aqueous humor were reduced in the eye receiving the dye. The oxygen uptake of the ciliary processes was reduced in only some instances.

3. These functions returned to normal at different times, the oxygen uptake recovering after about 96 hours, the intraocular pressure after 14 to 21 days, and the ascorbic acid remaining depressed for as long as four weeks.

4. The relationship of these observations to the effects of dye No. 350 on oxygen consumption and oxidation phosphorylation by certain cell-free tissue preparations is discussed.

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Discussion

Dr. Everett Kinsey (Detroit): Dr. Ballintine and Dr. Peters are to be complimented on their excellent method for determininng some of the more subtle features of the mechanism of secretion of aqueous humor.

With regard to attempting to determine which of the two alternative hypotheses suggested by Dr. Ballintine and Dr. Peters is the correct one, I would suggest that they inject animals with a tagged anion or some uncharged substance, since there is already good evidence that the mode of entrance of compounds of these kinds into the anterior chamber is chiefly by diffusion.

Another possible approach to the problem would

be to determine whether there is any change in the normally existing difference in pH of the aqueous humor and plasma. I assume that the difference in pH in these two fluids can be accounted for by the difference in concentration of total carbon dioxide present.

DR. FRANK A. VESEY (Schenectady): It seems to me that this paper is a very significant contribution to the clarification of physiologic aspects of the eye. I have been interested for a long time not only in the secretion of the aqueous but the excretion of the aqueous, which would elucidate to a great extent the much debated question of mechanical concept of glaucoma against the physiologic concept of glaucoma.

I would like to ask the author of this paper whether he did investigate the amount of dye entering into the aqueous, and I would very much like to suggest that he should inject this dye, if possible, into the anterior chamber, and then try to investigate the dye entering into the ciliary sinus of the rabbits.

Also, he should try to investigate the amount entering into the choroidal vessels and into the sheath of the optic nerve. I don't know to what extent this would be possible. Unfortunately, I can only think about these things, and I cannot do any actual investigation myself.

I would also like to suggest that Dr. Ballintine should try to obstruct the anterior chamber angle by mechanical means, and then see how the aqueous and the ciliary body and the other vessels of the choroid and adjoining vessels of the optic nerve will behave.

DR. ELMER J. BALLINTINE (Cleveland): In reply to Dr. Kinsey, I can only promise that we do contemplate attempting to answer the question of altered permeability by investigating further the entrance of foreign substances added to the blood stream and comparing the rates of entrance in the treated and untreated eyes. This will be done some time.

In answer to the question concerning entrance of these dyes into the aqueous: These are highly colored dyes. Their detection is easy and, in the experiments, we found no dye in the aqueous humor. On the other hand, the equatorial zones of the lens, in some experiments at least, were definitely stained by the dye an hour and a half after the injection; so, the dye must have gotten into the aqueous and then must have been absorbed by the lens.

I don't think any large amount ever gets into the aqueous humor, and I believe this is similar to what happens with the intravenous injection of many basic dyes. One of the things that characterizes basic dyes is that they stain the epithelium without entering the aqueous in large amounts.

We have performed a few experiments by simply adding the dye to the medium in which the processes were suspended, and not by injection into the aqueous humor itself, and the dye enters the process and stays isolated in the ciliary epithelium just the same.

The question of reversing the edema of the process by obstructing the outflow of aqueous humor, has not occurred to me, and I am certainly grateful for the suggestion.

COMPOSITION OF THE POLYSACCHARIDE OF THE LENS CAPSULE AND ITS TOPICAL DISTRIBUTION*

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New York

One of the causative factors considered as of possible major significance in the pathogenesis of certain forms of cataract is the permeability of the lens.[1a, b] Changes in this permeability can concern either the lens fibers or the epithelium, or the fibrous capsule surrounding the lens. It has recently been shown[2] that the latter possesses a considerable metabolism of its own, that it contains notable amounts of glutathione, adenosine triphosphate, and probably coenzymes I and II, and that it is able to oxidize glutathione and to break down anaerobically the pentose of nucleotides.

As, according to Harris,[3] there seems to be an intimate relation between the metabolism of the whole lens and its permeability, it seems possible that the lens capsule itself is involved in the regulation of the permeability of the lens and may depend in this function on the integrity of its metabolism and of its chemical composition.

Changes in the permeability of the capsule may depend on changes in either the chemical or physicochemical properties of its constituents. The main bulk of the latter is represented by proteins, which in some way are linked to a characteristic polysaccharide.

There is considerable evidence available that prosthetic groups of proteins like polysaccharides in glycoproteins, nucleic acids in nucleoproteins, or pigments in chromoproteins, are of decisive influence on the physicochemical properties of these proteins.

This suggested that changes in permeability of the lens capsule may be the expression of changes in the chemistry of its polysaccharides, which would in turn affect certain properties of its proteins. However, information on the chemical nature of the polysaccharide of the capsule has until recently been scanty and inconclusive.

After this investigation was started, A. Pirie[4] reported some results on the chemical nature of the polysaccharide of the lens capsules which was found by paper chromatography to consist predominantly of glucose, with a smaller amount of galactose. No decision was reached by this author with respect to the presence of hexosamine, and no reliable quantitative data were obtained as to the composition of the polysaccharide.

The constituent sugars were estimated in a preparation purified by adsorption on Zeo-Karb 215, which led to a loss of 50 to 60 percent of the carbohydrate. This loss could have selectively involved one of the constituents and might have changed the ratio between the various constituents. Furthermore, the method used for the determination of the composition of the polysaccharide was based on the carbazol reaction of sugars which is not able, because of the lack of sufficient specificity, to give adequately reliable quantitative results.

In our investigation, we determined in the first series of experiments, the nature of the constituents of the polysaccharide of the lens capsule of the beef, and determined its quantitative composition by a series of spectrophotometric methods recently elaborated which afford greater specificity and accuracy. The identification of the constituent sugars by paper chromatography was carried out after an extraction procedure which was shown not to change the ratio between two different constituents of the polysaccharide.

* From the Department of Ophthalmology, College of Physicians and Surgeons, Columbia University, and the Institute of Ophthalmology, Presbyterian Hospital. This study is a part of the work performed under Contract AT-30-Gen-70 of the Atomic Energy Commission.

Preparation of lens capsule

The eyes of freshly slaughtered beef were transported at low temperature and were used immediately after arrival in the laboratory. After the cornea was removed and a radial incision was made through the iris and ciliary body, these structures were ripped off to expose the zonula fibers, which were then severed around the lens.

Following separation of the lenses from the adherent vitreous body a cross incision through the capsule and cortical layers of the lens was carried out through posterior capsule and cortex. The nucleus of the lens and the lens cortex were removed carefully from the spread capsule preparation. The latter consisted then of the anterior and posterior part of the lens capsule and the intact lens epithelium.

In a series of experiments these preparations were weighed either individually or were collected in a weighing bottle in groups of about five to 20 and weighed. This material was either suspended in 5-percent trichloracetic acid or in 0.9-percent solution of sodium chloride, or was processed in the dry state.

In another experimental approach, the isolated lens was excised equatorially to separate the posterior from the anterior half of the lens capsule. Pooled preparations of the isolated posterior and anterior capsule in each group, or corresponding parts of individual capsules, were treated in accordance with the technique used in the first series.

In order to carry out the chemical examinations separately on the lens epithelium and lens capsule, the capsule was spread on a slide and the epithelium was removed by scraping with a scalpel. Examination in reflected light allowed close inspection of the surface of the membrane to ascertain the complete removal of the epithelium.

Finally, the central portion of the anterior lens capsule was severed from the peripheral ring zone of a width of about three mm. This separation of the equatorial and central portion of the lens capsule was carried out on capsule preparations with and without epithelium.

Analytical methods

Seven to 10 capsules were deproteinized by suspending them in five cc. of five-percent trichloracetic acid and repeatedly shaking for two hours at room temperature. The suspension was then centrifuged; the precipitate was washed twice with five cc. of five-percent trichloracetic acid and then suspended in five to seven cc. of half normal NaOH in which it dissolved, giving a slightly turbid solution.

The determination of hexoses was carried out on this solution by a series of three recently described reactions[8] with cysteine and sulfuric acid. In these three reactions, the three aldohexoses commonly found in animal and plant tissue, namely glucose, galactose, and mannose, produce colored products with characteristic absorption maxima.

In the first cysteine reaction,[8] which will be designated as cysteine reaction for hexoses, the absorption curves for all hexoses are completely parallel, giving a maximum between 412 and 415 mμ. Other classes of sugars give completely different absorption curves. By measuring the optical density at the maximum of 414 mμ and the absorption maximum at 380, we compensate any absorption due to other classes of sugars such as methyl pentose, pentose, and hexuronic acids which occur in polysaccharides, because in the test these sugars show identical absorption at these two wave lengths. On the other hand, the three aldohexoses mentioned above show differences in their extinction coefficients, that for galactose being only half as great as that for glucose.

In the second cysteine reaction,[8] designated here as cysteine reaction for galactose, the three aldohexoses produce a characteristic blue color with a maximal absorption at 605 mμ. The extinction coefficients for the three hexoses again differ, those for galactose, glu-

cose, and mannose being in the ratio 10:4:1 respectively.

Finally in the third cysteine reaction, the absorption curve for mannose is completely different from that for galactose and glucose, and the difference of the optical density at 400 mμ and 370 mμ for mannose is 10 times as high as for glucose and two and one-half times as high as for galactose. By making measurements in these three reactions, it is therefore possible to obtain three equations from which the concentration of all three hexoses, if present in the solution, can be quantitatively determined.

The precision of these determinations depends obviously on the magnitude of the differences between the extinction coefficients for the various hexoses. For a mixture of galactose or glucose and mannose, the precision is consequently much greater than for a mixture of galactose and glucose, as the differences between the extinction coefficients for the latter sugars in reaction 1 and 2 is not great. For this reason every determination was repeated at least once and in every determination triplicates of sample and internal standard were used. In this way it was possible to determine the ratio galactose to glucose within a limit of error of ± 10 percent.

That the determination of sugars by cysteine reactions in the polysaccharide does not require prior hydrolysis was shown in the following way: the solution of the polysaccharide was heated for two hours at 100°C. with 2 N H_2SO_4, after that time 90 percent of the total reducing sugar was liberated. The two cysteine reactions were carried out on the hydrolysate and on the nonhydrolyzed solution of the polysaccharide simultaneously. No differences in either the content of glucose and galactose by the hydrolyzed and nonhydrolyzed polysaccharide were found.

To check the results obtained with the cysteine reaction, the total hexoses were also determined by the anthrone[6] reaction. In this reaction the ratio of the extinction coefficients for glucose and galactose differs only by 10 percent from that in the cysteine reaction for hexoses.

For the detection of hexosamines,[7] the recently described very sensitive reaction with indole and HCl was used which allows the detection of as little as 5γ of hexosamines per cc. of solution. The test for hexuronic acids was carried out by the carbazole reaction,[8] correction being made for the amount of hexoses which was previously found by the cysteine reaction. Presence of keto sugars was tested by the recently described sensitive carbazole cysteine reaction,[9] for desoxypentose by the diphenylamine reaction,[10] and of methyl pentose and pentose by the characteristic reactions with cysteine and H_2SO_4.[11, 12] The determinations of nitrogen were carried out by micro-Kjeldhal.

The separation of the sugars of the polysaccharide by descending paper partition chromatography was carried out using a mixture of one part pyridine, one and one-half parts water, and three parts butanol, according to Chargaff.[13] The paper was dried after 24 hours and then again treated with the organic solvent used before. Complete separation between glucose and galactose was obtained in this way. For the detection of the spots, a spray of aniline phosphate according to Bryson and Mitchell[14] was used.

Results

The tests for methyl pentose, hexuronic acid, and hexosamine gave essentially negative results with the trichloracetic acid precipitation residue. As these tests detect 5.0 μg. per cc. of these compounds and the final dilution of the lens capsule was 34 fold with respect to the wet weight, it can be said that the lens capsule certainly contains less than 20 mg. of either hexosamine or hexuronic acid per 100 gm. of the wet capsule which contains about 1.0 gm. of polysaccharide.

The test for keto sugars, sensitive to 2.0 μg. per cc., was also negative. The only sugars found to be present in significant amounts in the capsule bound to proteins, were glucose and galactose.

Composition of the polysaccharide in the total lens capsule

A. Quantitative determination of hexoses

In Table 1 are listed the results of quantitative determination of glucose and galactose in the capsule. The optical density in cysteine reaction three (reaction for mannose) did not differ significantly from the value calculated from the concentrations of galactose and glucose, as obtained by the cysteine reactions one and two. The values for the two hexoses, therefore, in cysteine reactions one and two were calculated on the assumption that no mannose was present. The determination was carried out on several different batches of total lens capsules, every batch consisting of five to 20 capsules or parts of them.

In Table 1 are listed the total amounts of galactose and glucose in three batches of total capsules, and one batch of capsules dissected into the anterior and posterior part, and two batches of anterior capsules dissected into the equatorial and central parts. As can be seen, galactose is present in considerably higher concentrations than glucose, and the average value for the ratio of galactose to glucose from six experiments is 1.39, the individual values ranging from 1.24 to 1.53. The variations observed cannot be regarded as significant.

In experiment 2-b of Table 1, one batch of 10 eyes was left for five hours at room temperature and the eyes were dissected only after that period to test whether variations in the storing time of the eyes in the slaughter house could have had a significant effect on the ratio of galactose to glucose in the polysaccharide of the capsule. Although the value obtained on the stored eyes was the lowest of the whole series, the change was not regarded as significant.

In experiment number 3 (Table 1), the polysaccharide of the lens capsule was also determined by the anthrone reaction and the values expressed in terms of galactose. In this reaction the extinction coefficient for galactose is 0.55 of that for glucose, the value

TABLE 1

Ratio of galactose and glucose in the polysaccharide of the total lens capsule and of various parts of it

Experiment No.	Number of Capsules	Preparation	Galactose (in mg.)	Glucose (in mg.)	Ratio Galactose/Glucose	Remarks
I	10	Total capsule	2.59	1.89	1.37	
II a.	10	Total capsule *	2.63	1.85	1.44	Dissected immediately
b.	10	Total capsule	2.91	2.35	1.24	Dissected after 5h at R.T.
III*	11	Total capsule	3.02	2.18	1.39	
IV a.	18	Equatorial part	1.60	1.15	1.39	
b.	18	Central part	1.73	1.34	1.29	
V a.	18	Equatorial part	1.67	1.12	1.49	
b.	18	Central part	1.53	1.21	1.27	
VI a.	12	Anterior capsule	2.66	1.74	1.53	
b.	12	Posterior capsule	0.85	0.57	1.50	

* In this experiment the amount of total hexose was also determined by the anthrone reaction and expressed in terms of galactose.

obtained with the anthrone reaction, therefore expressed as galactose, should be about four-percent higher than the total value for hexose, expressed in the same way, found with the cysteine reaction. This was indeed the case. Furthermore it can be seen from Table 1 that the ratio of galactose to glucose is the same in various parts of the lens.

The total amount of polysaccharide was calculated as sum of the anhydro residues of galactose and glucose found with the cysteine reaction. This amount calculated on the basis of mg. per 100 gm. wet weight (Table 2), varied in the total capsule in four experiments between 880 and 915. The water content of the capsule may vary from one batch of the animals to the other because of differences of age or nutritional status, and may also depend on desiccation of the capsule during the preparation.

It therefore seemed necessary to calculate the content of the polysaccharide in percent of the proteins of the capsule. These values varied in four experiments between 8.6 and 9.9, the average being 9.2. Our values agree well with the values of about 10-percent obtained by other authors[4] who used the reducing power of the hydrolysate of the lens capsule as a measure of the amount of polysaccharide.

B. Demonstration by paper chromatography of galactose and glucose as the main constituents

To carry out partition paper chromatography of the sugars of the polysaccharide, it was necessary to separate the polysaccharide from proteins without changing its composition. This could be achieved by extracting the lens capsule first with five-percent trichloracetic acid at room temperature for two hours, centrifuging off the supernatant, washing the precipitate twice with five-percent trichloracetic acid and extracting the precipitate at 90°C. with five-percent trichloracetic acid for two hours, renewing the trichloracetic-acid solution every half hour. Under these conditions more than half of the total polysaccharide of the capsule is extracted while only small amounts of protein go into solution.

The ratio of galactose and glucose found in this procedure was not significantly different from that in the capsule itself. The trichloracetic-acid filtrate was heated for three and one-half hours under reflux to destroy trichloractic acid, and then for one hour with 2 N H_2SO_4 which was found sufficient to yield 90 percent of the total reducing sugar. The hydrolysate was brought up with $Ba(OH)_2$ to pH 6.0 and evaporated in vacuo to dryness.

The dry residue prepared from 18 lens capsules, weighing 1.05 gr., was finally dissolved in 0.5 cc. of water and 6.0 µl of this solution was used for paper chromatography. As standard, 2.0 µl of a solution containing 20 µg. glucose and 20 µg. galactose were used. The chromatogram is shown in Figure 1.

As can be seen, two spots were obtained from the lens capsule extract which correspond exactly to the position of the two sugars in the standard. The spot corresponding to galactose is considerably more intensely colored than that of glucose, whereas in the standard solution the two spots appear as equal in intensity. This confirms the results of the quantitative determination of the ratio of galactose and glucose.

To ascertain further the quantitative relation between galactose and glucose, the spots on the paper chromatogram were eluted with water filled up to the same volume and the amount of sugar in both spots compared with the anthrone reaction. The ratio between the spot for galactose and that for glucose obtained in this way was 1.65, which appears in good agreement with the ratio obtained by the cysteine reaction on the total polysaccharides.

Distribution of polysaccharide in various parts of the lens capsule

As the equatorial and central parts of the anterior capsule and the posterior capsule

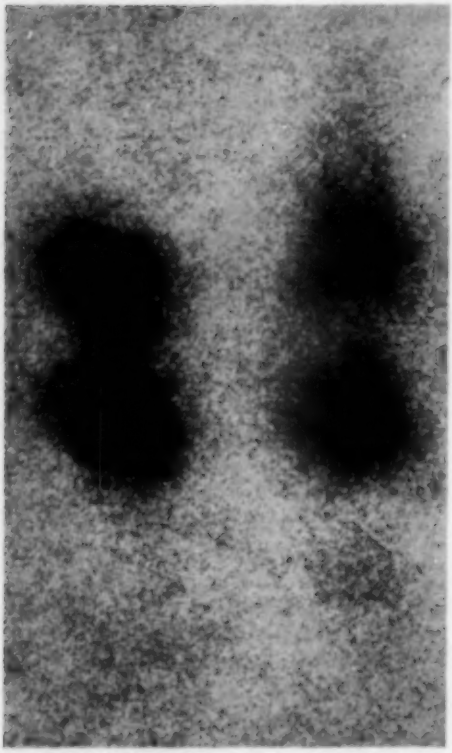

Fig. 1 (Dische and Borenfreund). Chromatogram. Left upper spot 20-micrograms glucose standard. Left lower spot 20-micrograms galactose standard. The two spots on the right-hand side were obtained from the hydrolysate of the capsule.

may differ in their functional significance, we determined in a series of experiments the content in polysaccharide of these three separate parts of the lens capsule. The ratio of weights of the equatorial and central part of the interior capsule varied between 1 : 1 and 2:1, which corresponds to variations of the radius of the central part between 0.6 and 0.67 of the radius of the total anterior capsule.

Total hexose was determined in each part either by the cysteine reaction for hexose or by the anthrone reaction, and the total polysaccharide was calculated on the basis of the ratio of 1.4 to 1.0 which was found in previous experiments, and subtraction of 10 percent of the total weight of the individual sugars to obtain the weight of the anhydro residues.

As can be seen in Table 2, the concentration of the polysaccharide calculated in percent of protein in all experiments varied in the equatorial part of the anterior lens capsule between 7.2 and 9.6 with an average from five experiments of 8.4, and between 8.8 and 12.5 with an average from five experiments of 10.3 in the central part.

It should be noted that the lower value of carbohydrate in the equator was obtained in every one of the five individual cases. Furthermore, since the protein content of the equatorial part was always lower than in the central part, the ratio of the concentrations of the polysaccharide in the equatorial and in the central part expressed in mg. per 100 gm. wet weight is still much lower, varying in the first between 700 and 960 with an average of 800 and in the latter between 1,210 and 1,980 with an average of 1,450 from five experiments. The concentration in the posterior lens capsule on the other hand was not found significantly different from the concentration in the total anterior capsule whether calculated by wet weight or in percent protein.

Discussion

The polysaccharide of the lens capsule was found in our experiments to consist of galactose and glucose in such proportion that for about two molecules of glucose, three molecules of galactose are present. This predominance of galactose appears also confirmed by the results of the chromatographic separation of the two hexoses. These results seemed at variance with the results of Pirie who found predominantly glucose as a constituent of the lens polysaccharide. This disagreement can be explained by the fact that Pirie purified the hydrolysates of the polysaccharide with Zeo-Karb, which procedure involved a loss of about 40 to 50 percent of the total sugar. It could be that this

TABLE 2

POLYSACCHARIDE CONTENT OF THE TOTAL LENS CAPSULE AND ITS VARIOUS PARTS

Experiment No.	Number of Capsules	Preparation	Wet Weight (in mg.)	Protein in % Wet Weight	Total Polysaccharide	
					In mg. per 100 gm. wet weight	In % of Proteins
I	10	Total capsule	460	9.9	880	8.9
II	10	Total capsule	424	10.5	910	8.6
III	11	Total capsule	530	9.0	890	9.9
IV	5	Total capsule	257	9.8	915*	9.3*
V	7	Total capsule	327	8.8	800*	9.1*
VI a.	10	Total anterior capsule	395	10.2	940	9.1
b.	10	Posterior capsule	145	4.65	560	12.0
VII	8	Posterior capsule	75	7.4	760	10.4
VIII 1)	10	Anterior capsule, equatorial part	282	8.0	700	8.8
2)	10	Anterior capsule, central part	135	12.2	1515	12.5
IX 1)	10	Anterior capsule, equatorial part	239	10.0	715	7.2
2)	10	Anterior capsule, central part	115	13.6	1210	8.9
X 1)	12	Anterior capsule, equatorial part	192	10.6	936	8.8
2)	12	Anterior capsule, central part	160	15.6	1512	9.7
XI a. 1)	10	Anterior capsule, equatorial part	253	10.0	965	9.65
2)	10	Anterior capsule, central part	102	14.0	1600	11.4
b.	10	Posterior capsule	98	5.85	665	11.4
XII a. 1)	9	Anterior capsule, equatorial part	230	9.8	730	7.5
2)	9	Anterior capsule, central part	99	16.2	1430	8.8
b.	9	Posterior capsule	105	5.0	535	10.7

* Determination of the polysaccharide carried out with the anthrone reaction—all the others with the cysteine reaction.

loss was due to a predominant absorption of galactose by the absorbent.

The most significant result of the present investigations seems to be the fact that the concentration of the polysaccharide varies in various parts of the lens capsule. These variations concern not only the absolute amount of the polysaccharide but also its ratio to the protein content. Thus, while the concentration of the protein in the equatorial part of the capsule, which in our experiments corresponded to about 0.3 to 0.4 of the outer radius of the anterior capsule, is by one-fourth to one-third smaller by wet weight than in the central part, the concentration of the polysaccharide in some cases is less than one half as great in the periphery than in the center, and in the average amounts only to 55 percent of the value in the center.

That means that the protein in the periphery is combined with much less of the polysaccharide than the protein in the center. If we assume, as seems reasonable, that the concentration of the polysaccharide in the proteins decreases continuously from the poles to the equator of the lens capsule, it becomes clear that the concentration of the polysaccharide in the immediate neighborhood of the equator may be very low or perhaps even zero.

The variations in the content of the proteins in polysaccharide are larger than could be expected from the limit of error of the methods used for the determination of the proteins in the carbohydrates. These variations may be ascribed partly to the possibil-

ity that either some fibers in an uncontrollable way remained on the capsule or that the capsule contained some protein to which no carbohydrate was attached, and which can be more easily pressed out of the capsule by the mechanical pressure used in stripping off the remnants of fibers.

There can be little doubt that the polysaccharide attached to the protein, which amounts to about 10 percent by weight of the protein, may influence to a considerable extent the physicochemical properties of the protein, and it seems quite possible that these physicochemical properties are in some way correlated to the permeability of the lens capsule. It is therefore necessary to consider the possibility that on the one hand the equatorial part of the capsule which contains less of the polysaccharide may possess a different permeability as compared with the central parts, and that changes in this permeability may affect the metabolism of the lens.

This possibility could prove pertinent to the question of the origin of certain types of cataract, particularly the galactose and diabetic cataract. In the case of galactose cataract, it was shown that apart from galactose, xylose is able, when fed in comparable quantities, to produce an identical disturbance in the lens. Xylose is structurally related to galactose insofar as carbons 3 and 4 are at cis position in the two sugars, which is not the case with glucose or another pentose, arabinose, which produces cataract if at all, only when used in much larger quantities.

This fact clearly suggests that one at least of the pathogenic factors in galactose cataracts may be interference with the metabolism of galactose which can be induced either by too high concentrations of this latter sugar, or by inhibitory effects of a sugar of a similar configuration. Such interference could be directed either against a normal production of the polysaccharide or affect its distribution in various parts of the lens which could be significant for the lens from the nutritional point of view. Changes in the composition or distribution of the polysaccharide, however, could also be at least one of the causative factors in the pathogenesis of other forms of cataract.

The presence of galactose may indicate certain similarity to polysaccharides found in the nervous tissue. In this connection, it seems interesting that the lens capsule represents a fibrous membrane derived not from connective tissue, but from the lens epithelium. It therefore seems possible that further investigations may establish similarities in the chemical composition of the lens polysaccharide with polysaccharides in other membranes of a similar origin like basal membranes separating other epithelial formations from the connective tissue or extracellular fluid.

Our experiments bring no evidence that there is only a single polysaccharide consisting of galactose and glucose. It seems quite possible that two different polysaccharides are present—one being a polymer of galactose and the other of glucose. In any case, however, this polysaccharide (or polysaccharides) represents a type of saccharide which has not yet been found in other animal tissue.

Summary

1. The bovine lens capsule contains a polysaccharide which consists of galactose and glucose, the ratio of the first to the second being approximately 3 : 2. No significant differences in the composition of the polysaccharide were found between various parts of the capsule.

2. The polysaccharide is combined with the protein of the capsule and represents about 10 percent of the total protein of the capsule.

3. Marked differences in the topical distribution of the polysaccharide in various parts of the lens capsule were found. The content of the protein in the central part of the capsule in polysaccharide is higher than in the equatorial part. As the concentration of the protein is also higher in the central than in the equatorial part, the concentra-

tion of the polysaccharide by wet weight in the equatorial part amounts only to one half to two thirds of its concentration in the central part.

4. The possible significance of these facts for the pathogenesis of certain forms of cataract is discussed.

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THE NUTRITIONAL SUPPLY OF CORNEAL REGIONS IN EXPERIMENTAL ANIMALS*

III. Further studies on the corneal transport of inorganic ions

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In the preceding paper of this series,[1] it was demonstrated that the corneal endothelium is highly permeable to inorganic ions in both directions and it was suggested in agreement with others[2,3] that secretion of inorganic ions into the aqueous might be the osmotic pump which maintains corneal dehydration and transparency.

Data presented at that time were not considered adequate to warrant definite conclusions on this point. The present report represents an extension of the previous experiments in an attempt to clarify this central problem of corneal transparency.

If either epithelium or endothelium were actively transporting ions (and causing dehydration by osmotic force) chemical or physical damage to these layers should drastically alter the rate of transfer. The experiments reported here largely concern the fate of intracorneally injected $Na_{22}Cl$ in normal and damaged eyes. One set of experiments follows the fate of intracamerally injected $Na_{22}Cl$ after damage to the corneal endothelium.

Methods

Healthy adult albino rabbits under nembutal phenobarbital anesthesia were used in all experiments. Injection both intracorneally and intracamerally was made with the microsyringe and by the technique previously described.[4] The solution of 1.35-percent NaCl was held on the epithelial surface in the suction applicator previously described. In one series of experiments a flow applicator was used (fig. 1) through which 40 cc. of 1.35-percent NaCl was passed over the epithelial surface during the 20-minute experimental period.

In all experiments the injected solution was 1.3 mm.^{3} of $Na_{24}Cl$ containing 10 $\mu c Na_{24}$† made isotonic with NaCl and isohydric with a small quantity of phosphate buffer. Under our conditions of counting with a thin mica windowed GM tube this total activity yielded 2,000 to 3,000 counts per minute and allowed one percent of the total dose to be detected with assurance.

The epithelium was removed where so indicated by rubbing with a cotton applicator moistened with a 10-percent solution of sodium desoxycholate.[5] This removal was completely efficient and did not unduly traumatize Bowman's membrane.

The endothelium was abraded by the method of Cogan.[6] A 1 by 1 by 2-mm. piece of alnico V was implanted in the anterior chamber through a small incision and two or more days later just before injection of the tracer solution the tiny magnet was dragged over the endothelial surface in the field of the Parker hand electromagnet for a period of approximately 10 minutes.

That the endothelium was abraded could be easily determined in the slitlamp and was borne out by clouding and increase of weight of the cornea (see later). Quantitatively, however, the procedure was not as completely reproducible as would be ideally desired and numbers of animals were required to give a reliable mean figure.

Chemical damage was attempted by application of 0.23 M Iodoacetate to the anterior

* From the Laboratory for Research in Ophthalmology, Western Reserve University and Ophthalmologic Service, Department of Surgery, University Hospitals of Cleveland. Supported by a grant from the National Institute of Neurological Diseases and Blindness, United States Public Health Service.

† Na_{24} was obtained from the Oak Ridge National Laboratory, Oak Ridge, Tennessee.

corneal surface on a cotton sponge either with or without mechanical damage to the epithelium. Application of 0.2 M NaCN was performed similarly. In one set of experiments iodoacetate was injected intralamellarly together with the tracer salt. In still another set the tracer was injected 18 hours after replacing the aqueous humor with a 0.3 M solution of alloxan—similar to Langham.[7]

Results

A. Time course

The time course of distribution of intracorneally injected sodium is shown in Figure 2. As one might expect the content of the aqueous passes through a maximum while that of the cornea steadily declines. In the short time experiments it can be noticed that recovery is quite good if only cornea and aqueous are considered. Later loss by aqueous outflow causes recovery to fall off.

If one considers time course of concentration (fig. 3) it is evident that the corneal Na_{22} concentration falls in the logarithmic fashion expected, and that the aqueous passes through a maximum. However most remarkably after the first 20 minutes the aqueous concentration of sodium is higher than that of its source.

This will be considered in more detail in the discussion below.

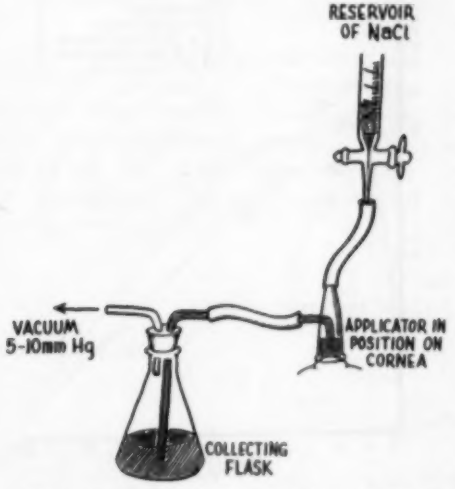

Fig. 1 (Potts, Goodman, and Johnson). Flow applicator for anterior corneal surface.

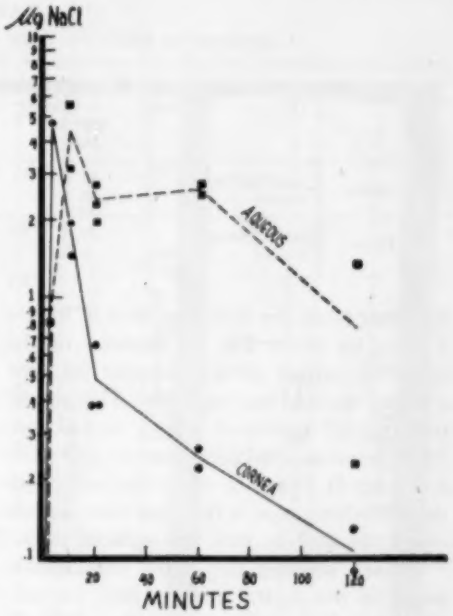

Fig. 2 (Potts, Goodman, and Johnson). Time course of Na_{22} distribution after intracorneal injection.

B. Endothelial damage

The amount of sodium as percent of total dose after intracorneal injection is shown in Figure 4 for four types of samples. The experimental eyes have had their endothelium denuded with the magnetic flea just before the injection. The control eyes are undamaged. Each line on the graph represents the figure for one eye, the diamond shows the mean value and the arrows indicate the standard error of the mean. It can be seen by inspection that there is no significant difference between the control series and the experimental series when endothelium is damaged.

C. Epithelial damage

When one considers epithelial damage on

TABLE 1

COMPARISON OF STATIC AND FLOW APPLICATORS—EPITHELIUM REMOVED

		Percent Total Dose				
		Applied Soln	Aqueous	Cornea Ring	Cornea Plug	Total
Static	Experimental	35.5	6.8	0.38	0.69	43.4
	Control	10.8	31.3	1.26	8.60	51.9
Flow	Experimental	20.4	5.2	0.04	0.64	26.3
	Control	6.0	33.6	0.95	9.21	49.8

the other hand, the data presented in Figure 5 leave no doubt that the removal of the epithelial barrier allows diffusion into the artificial tears of the applicator. This simply confirms the results of Kinsey and Cogan. There is no unusual concentration phenomenon here. It should be noted that even with the epithelium gone in this case more sodium enters the aqueous than the artificial tears.

To see whether this latter phenomenon might be due to the aqueous flow, we substituted a flow applicator for the static applicator ordinarily used and, as seen in Table 1, this proved not to be a significant factor. The results with either applicator are the same.

D. Damage to epithelium and endothelium

Figure 6 shows a similar set of experiments where both surfaces of the cornea were damaged. It is evident that the experimental eyes are markedly different from the controls. It is equally evident that all the difference is due to the epithelial damage since these eyes with both membranes damaged do not differ significantly from the epithelium damaged eyes.

Table 2 indicates the mean increase in weight of the corneal portions after damage to epithelium, endothelium, and to both layers.

E. Chemical damage

All of the attempts at chemical damage with iodoacetate and cyanide were fruitless. Not only did they create no differences between experimental and control eyes, but there was no evidence of corneal clouding or increase in weight of the center plug.

Only in the eyes where alloxan was injected into the anterior chamber did any opacity or increase in weight take place. Eighteen hours after alloxan the cornea plug had increased its weight by 100 percent. The mean values for three alloxan experiments are indicated in Figure 2. It is evident that there is no significant decrease in aqueous concentration caused by alloxan poisoning.

F. Dead animals

In one additional series the intracorneal

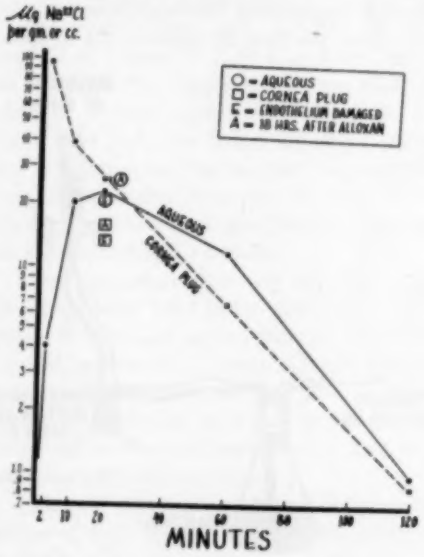

Fig. 3 (Potts, Goodman, and Johnson). Time course of $Na_{22}Cl$ distribution after intracorneal injection.

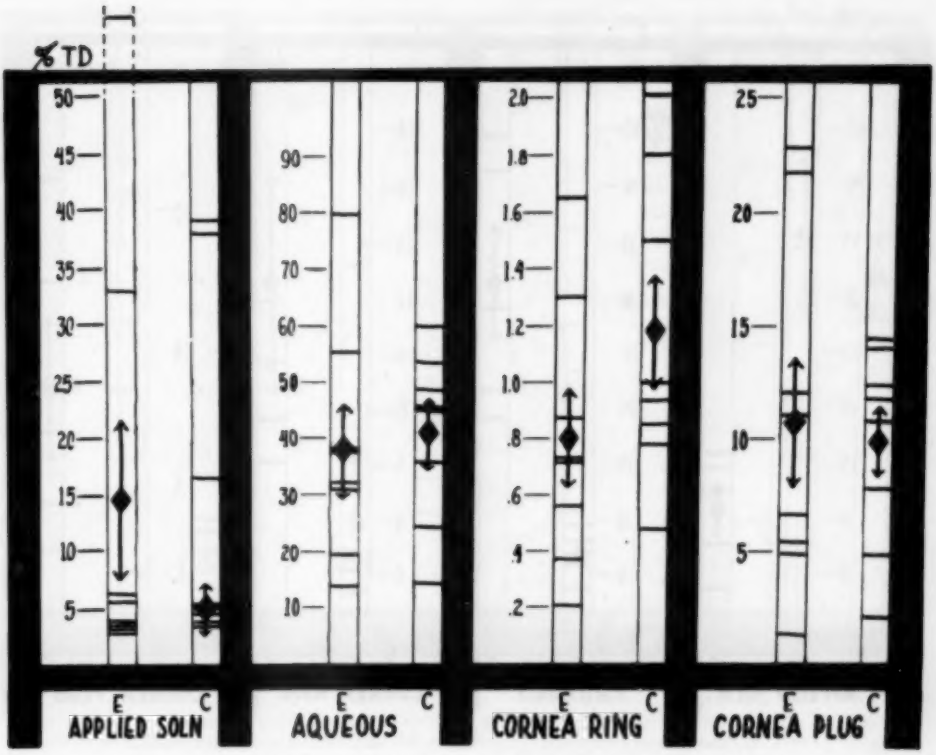

Fig. 4 (Potts, Goodman, and Johnson). Distribution of intracorneally injected $Na_{22}Cl$—endothelium damaged. (Time, 20 minutes; Experiments 379-387.)

injection was done in an animal which had been killed with an overdose of nembutal just previously. These results are shown in Table 3. It is seen that recovery is quite high because of the lack of aqueous outflow but large amounts of Na_{22} are found in the aqueous nevertheless.

Discussion

The behavior of sodium concentration is of particular interest. After the first 20 minutes the rising aqueous concentration and the falling cornea concentration cross and from this time on the aqueous humor has the higher sodium concentration. When one

TABLE 2

Increase in weight of corneal plugs after damage

Area = 3.14 cm.²
Diameter = 10 mm.

	Number of Eyes	Mean Weight Experimental	Mean Weight Control	Mean Increment
Epithelium Damaged	5	61.2 mg.	47.3 mg.	13.9 mg.
Endothelium Damaged	15	67.8	42.3	25.5
Both Damaged	8	70.5	46.3	24.3

Mean weight of all controls = 44.1 mg.

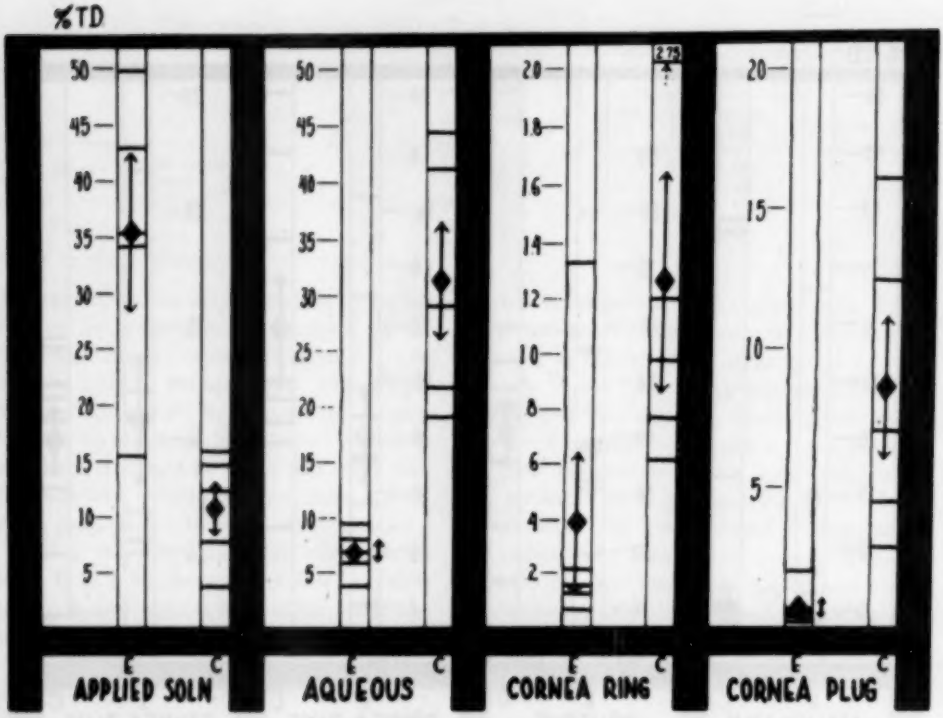

Fig. 5 (Potts, Goodman, and Johnson). Distribution of intracorneally injected $Na_{22}Cl$—epithelium damaged. (Time, 20 minutes; Experiments 370-374.)

adds to this the fact that when sodium is injected intracorneally the corneal concentration never reaches the aqueous concentration,[1] the suggested conclusion is that sodium is being secreted into the aqueous. All of the herein described corneal concentrations are, however, calculated on the basis of total weight of cornea. If one calculates these concentrations on the basis of total water or extracellular water, the concentration goes up correspondingly and the evidence for secretion is correspondingly less conclusive.

It would be convenient to be able to add that water is being carried along by osmotic forces and so is being secreted indirectly. However, the evidence of the endothelial

TABLE 3

DISTRIBUTION OF INTRACORNEALLY INJECTED $Na_{22}Cl$ IN LIVING AND DEAD EYES—EPITHELIUM REMOVED

		Percent Total Dose				
		Applied Soln	Aqueous	Cornea Ring	Cornea Plug	Total
Living	Experimental	20.4	5.2	0.04	0.64	26.3
	Control	6.0	33.6	0.95	9.20	49.8
Dead	Experimental	70.7	19.6	0.94	2.61	93.8
	Control	12.0	57.2	1.47	18.1	88.8

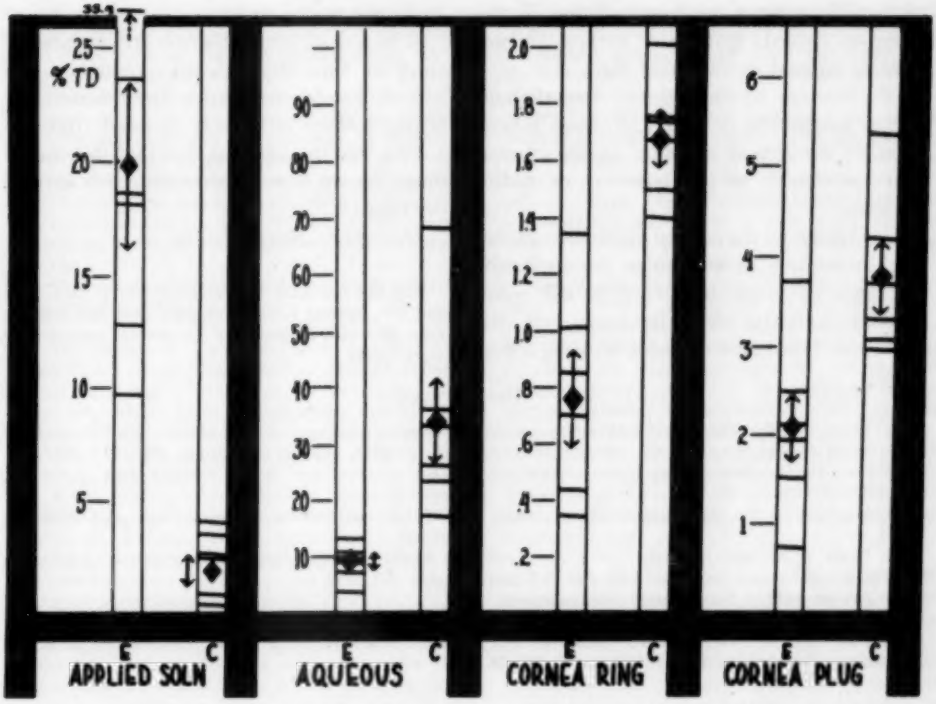

Fig. 6 (Potts, Goodman, and Johnson). Distribution of intracorneally injected $Na_{22}Cl$—epithelium and endothelium damaged. (Time, 20 minutes; Experiments 395-403.)

damage experiments, whether with the magnetic flea or with alloxan, indicates that water movement is independent of sodium movement.

In both of these types of experiments the sodium movement is unaffected by the endothelial damage even though water movement obviously is affected and corneal hydration and clouding take place. Thus, sodium movement and corneal dehydration do not appear to be connected.

The experiments on damage to epithelium alone and to both epithelium and endothelium demonstrate what was known before: that epithelium is relatively impermeable to sodium ion. It is interesting to note, however, that even when epithelium and endothelium are both damaged there is not the even distribution on either side of the cornea that one might expect. Even here only 30 percent or so of the salt (less with the flow applicator) finds its way to the artificial tears. Again, this not the type of partition expected from diffusion alone, but an alternative mechanism is not obvious, and in any case must be independent of water movement.

In order to acquire additional information on possible mechanisms of ion transport and their possible connection with corneal hydration, experiments are in progress with other ions both positive and negative and with tritium labeled water. As yet these experiments are in too early a stage for discussion.

Summary

1. After intracorneal injection of $Na_{22}Cl$ the sodium ion concentration of the cornea falls logarithmically and the concentration in the aqueous passes through a maximum.

After 20 minutes the concentration in the aqueous exceeds that in the cornea, but not that in corneal extracellular fluid.

2. Damage to the corneal endothelium either mechanical or chemical causes inhibition of water and clouding of the cornea, but no change in the behavior of radio-sodium.

3. Damage to the corneal epithelium causes significant loss of sodium to the applicator of artificial tears, but there is still more sodium entering the circulation via the aqueous. This is not changed by using a flow applicator on the cornea.

4. In a dead animal there is still significant entry of Na_{24} from cornea to aqueous.

5. A possible conclusion from these facts is that sodium is actively secreted from the cornea into the aqueous, but that this movement has no direct connection with corneal dehydration.

University Hospitals (6).

The authors wish to thank Dr. David G. Cogan and Dr. Everett Kinsey for their pertinent suggestions on which a number of the present experiments were based.

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Discussion

Dr. John E. Harris (Portland, Oregon): I would like to interject a thought that perhaps is not inappropriate, particularly with reference to what has been said to be a divergence of data between Dr. Cogan and Dr. Kinsey on the one hand and of Dr. Potts and his associates on the other.

Not too many years ago we were in a somewhat comparable position with respect to the erythrocytes and other cells. For years it was well known that the erythrocyte was permeable to anions, and it was pointed out that of necessity the erythrocyte membrane must be impermeable to the positive ions or else it would swell and rupture when placed in sodium chloride solution. Analytical data in which the cation content was measured after placing these cells in various media designed to upset the cation ratio supported this view.

Then, when radioactive isotopes became available, it was found that in reality the erythrocyte did pick up radioactive potassium and radioactive sodium. This necessitated the postulation of some active transport mechanism which, as a matter of fact, was demonstrated at about the same time. Thus, far from being impermeable, an exchange of cations does occur across the erythrocyte barrier to a limited degree but an active process tends to maintain a constant composition. To the extent that the composition is not easily altered then, the membrane may be considered to be effectively impermeable to cations. While the conclusions from the two sets of data are quite opposite they were based on the best available evidence at the time and represent the only reasonable interpretation.

I think the same situation obtains for the cornea, although I may be oversimplifying it since the anatomic structure and chemical composition are quite different. Dr. Cogan and Dr. Kinsey came up with the only logical explanation of the data they had—that by analytical measurements they could detect no mass movement of electrolyte into or through the cornea. Using marked ions, however, it has been shown by Dr. Potts and his associates and others that radio-sodium does traverse the endothelium and to a lesser extent the epithelium. Under normal circumstances the sodium which enters the cornea by a variety of routes is compensated by an equivalent loss of sodium by excretion across the corneal surfaces, particularly the endothelium.

We have then effectively impermeable membranes that will respond to an osmotic gradient. Thus, both investigators have come up with somewhat the same concept on perfectly valid evidence, but from a different point of departure.

I was interested in Dr. Potts' observation that corneal hydration increased when he injured the endothelium, but yet the entrance of radioactive

sodium was no more rapid. I think this brings up one very good point—that actually the dehydration of these systems does not depend solely on the cation or anion balance.

We have been able to show several times an increase in the total osmotic activity as measured by the sum of sodium and potassium in the lens, in the presence of an actual dehydration of the lens. This also has been shown to be true in liver cells. In both the lens and liver cells, those factors which seem to promote a cation transport in some manner promote a dehydration of those particular structures.

Perhaps the hydration of the cornea is influenced in a similar manner.

Dr. George K. Smelser (New York): I must say that I am very interested in these experiments that have been related to us by Dr. Potts, and I am in complete agreement with him that his story of the nature of the maintenance of transparency in the cornea is an old one and not yet completely solved.

I was quite sure that the role of metabolism in maintaining transparency was definitely established a short time ago. At the same time I think we should not forget that this older concept, which was elaborated upon by Dr. Cogan and Dr. Kinsey, should not be entirely forgotten.

If you can show that the transparency of the cornea is interfered with when you interfere with metabolic activity, you can also show at the same time that if you change the osmotic pressure of the free cornea film by drying that film you also will introduce a factor which controls this dehydration and therefore the transparency. So, if the story is to be one of transport of these ions, as I think it is, it is also one which is involved in osmotic relationship of the precorneal tear film.

Recently I had a communication from Lyman, and he is now of the opinion that it might be an active ion which is actively transferred and as a result is the cause of the dehydration.

Dr. Everett Kinsey (Detroit): Evidence is accumulating that there is an active transfer of sodium from the stroma through the endothelium, and to a lesser extent through the epithelium, of the cornea. It seems possible that the primary mechanism of this transfer, or secretory activity, may be the same as that which we have hypothesized for the ciliary body, namely that hydroxyl ions are produced on the endothelium or epithelium in the same manner as hypothesized by Friedenwald for the epithelium of the ciliary body. On these limiting membranes the hydroxyl ions are converted to bicarbonate ions by combination with carbon dioxide. The negative biocarbonate ions on the surface of these membranes then attract cations, such as sodium, which diffuse into the aqueous humor and/or tears.

The similarity of the existence of enzyme systems in the stroma and epithelium of the ciliary body and the stroma and epithelium (and endothelium) of the cornea suggests that the hypothesis may be the correct one. Dr. Friedenwald and associates have shown that the epithelium contains a full complement of enzymes which are necessary for glycolytic and respiratory activity, whereas the stroma contains enzymes, dehydrogenases, which are associated with glycolysis. This differential type of distribution of catalysts gives rise to a transfer of electrons from one tissue to another, with the consequent formation of hydroxyl ions.

It is of some interest, too, I believe, that the pattern of metabolic activity for the lens also appears to be similar to that of the ciliary body and cornea. Dr. Frohman and I recently showed that the carrier necessary for respiratory activity, namely, cytochrome system, appears to be limited to the epithelium, whereas the stroma contains only those enzymes necessary for glycolysis.

Whether the similarity of chemistry of the lens and ciliary body also means that the lens epithelium functions to transfer substances actively into the lens remains to be demonstrated, but the possibility certainly exists.

Dr. Albert M. Potts: I would like to thank the discussers for their comments. They were all highly pertinent. There are only a few specific items that I would like to mention.

In regard to the connection between osmotic balance and corneal transparency, I think it is generally agreed that hydration and corneal transparency run hand in hand, that osmotic balance and hydration can be expected to run hand in hand, and that although there may be temporary perturbations of the system, those parallelisms can be expected to be maintained over a long period of time. Certainly, in our experience, wherever there has been a change in transparency, there has been an increase in hydration roughly proportional to the change in transparency.

As far as the osmotic mechanisms are concerned, we are not to a point where we can state definitely what their connection is.

In regard to the corneal tear film as mentioned by Dr. Smelser, one caution ought to be interjected, and that is to differentiate between the change in transparency caused by hydration, and loss of the iso-indicial nature of the corneal material, and that caused by possible roughening of one of the corneal surfaces, which is a different but also a possible method for causing change of transparency.

Dr. Kinsey, in the course of his remarks, mentioned the anions that interest us: bicarbonate (secondary to his work on the ciliary body) chloride, of course, because of its high concentration, and a number of organic anions of which lactate is a very good example.

THE ROLE OF METABOLISM IN THE HYDRATION OF THE ISOLATED LENS AND CORNEA*

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INTRODUCTION

A fundamental property of both lens and cornea is their transparency. This property depends on the optical homogeneity of the tissue, but the exact mechanism whereby this homogeneity is maintained is still debated. Characteristically lens and cornea are compact cellular structures with minimal extracellular space. Changes in structure or in the intra- or extracellular space of these tissues by the inclusion or formation of substances with different indices of refraction will result in a relative degree of opacity. One such substance commonly found is water. An increase in the water content is therefore of prime importance in reducing the transparency of lens and cornea.

The water content of tissues and more specifically of cells is kept at a constant level. Lens[1] and cornea[2] have been shown to maintain constant water contents. With a change in water content, there has been observed an associated simultaneous shift in the normal intra- and extracellular water and ion distribution.

Cellular water and ion distribution had been thought previously to be regulated by purely passive phenomena such as osmotic equilibria and membrane impermeability. Recently evidence has been accumulating which points to active metabolic processes of the cell controlling both the water content[3] and ion distribution.[4-6]

The existence of regulating mechanisms for ions and water dependent upon cellular metabolic activity are thus indicated. An ion regulating mechanism has been postulated as the sole controlling factor for cellular water. Whereby failure of this process then allows the cellular proteins to exert their potential osmotic pressure with a consequent water shift. Other theories favor the existence of separate mechanisms for ions and water or one common controlling process for both. This problem is still largely a matter of speculation.

This investigation therefore has attempted primarily to determine whether the maintenance of the water content of lens and cornea requires energy produced by the metabolism of the tissue.

The first consideration is whether energy is required to keep water out of the tissues or prevent its hydration—then whether energy is required to keep water in the tissues or prevent its dehydration—and, that once water passed in or the tissues become hydrated, whether metabolic energy was required in the removal of this water. Finally some energy production mechanisms that the tissue could resort to for the provision of increased amounts of metabolic energy for its water maintenance were studied.

MATERIALS AND METHODS

The technique in essence consisted in investigating the weight change of the isolated tissue in isotonic buffered salt solutions under varied conditions. An attempt was made to maintain physiologic conditions by obtaining (1) asepsis, (2) a solution approximating content and tonicity of aqueous, (3) a constant pH, and (4) a constant temperature by incubation at 37.5°C. In this way tissues could be studied in an approximate physiologic steady state.

The bovine lens was used for all studies on lens. These were obtained four to five hours

* From the Cellular Physiology Laboratory, Departments of Ophthalmology and Physiology, College of Medicine, State University of Iowa. This study was aided by a grant from the John and Mary R. Markle Foundation.

after enucleation from the abattoir* and arrived packed in ice. The lens was removed intact by an anterior approach, all precautions taken to avoid contamination. All lenses with ruptured or punctured capsules were discarded.

Initially, in our experiments on cornea an attempt was made to use bovine cornea but it was found that contamination could not be avoided. The use of antibotics, it was believed, would invalidate results by interference with the metabolism of the tissue.

Therefore rabbit corneas were used. They were obtained immediately after the death of the animal by intravenous air injection. The cornea was removed by an extralimbal incision. Precautions were taken to avoid damage to the cornea, as any injury within the limbus would be soon evident by immediate swelling in the physiologic saline solution.

The experimental solution used was an artificial aqueous solution devised by Merriam and Kinsey.[8] The lactic acid and urea ingredients however were omitted.†

The solution was prepared and autoclaved as required without the sodium bicarbonate and glucose. These were sterilized separately by passage through a Selas candle and then added. Five-percent carbon dioxide in air was then bubbled through the final solution for 20 minutes to obtain a pH of 7.40 with a variation of plus or minus 0.02.

The glucose content was 80 mg. percent and the tonicity approximately 152 milliosmoles.

In order to maintain a constant pH over the experimental period, usually 48 hours for the lens and 24 hours for the cornea, it was necessary to utilize 500 cc. of this solution for the bovine lens and 50 cc. for the rabbit cornea. In our preliminary experiments there was great difficulty in maintaining a constant pH of the solutions, the pH being easily overcome by the metabolic activity of the tissue. Three hundred cc. of Krebs-Ringer bicarbonate solution (approximately one half the buffering capacity of the Merriam-Kinsey solution) containing a bovine lens was not sufficient to maintain a constant pH over a 48-hour period. Similarly with a rabbit lens over a 24-hour period and 15 cc. of Krebs-Ringer bicarbonate solution, there was a pH shift of 7.60 to 7.34 and with 15 cc. of the Merriam-Kinsey solution a shift of 7.48 to 7.27. The rabbit cornea in 15 cc. of these solutions showed a similar shift in pH.

The use of a bicarbonate buffer required an anaerobic determination of the pH of the solutions. This was done by the hypodermic glass electrode. There was usually no more variation than 0.02 in the pH readings. Readings were determined initially and finally on the experimental solutions.

The lens and cornea were handled with aseptic technique—the greatest possible precautions taken to maintain sterility. The tissues were removed in a relatively sterile tissue culture room. Caps and masks and sterile instruments were used. After removal, both lens and cornea were gently blotted dry and weighed on a torsion balance with an accuracy of 2.0 mg. The tissues were then placed in the sterile solutions in 500 cc. and 125-cc. flasks for lens and cornea respectively. The flasks were sealed with rubber sleeves and then incubated at 37.5°C. till the end of the experimental period.

Observations on the appearance and transparency were made on the lens at 24-hour intervals and the cornea at 12-hour intervals. At the end of the experimental period usually 48 hours for lens and 24 hours for the cornea, samples were withdrawn through

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* Wilson and Company, Cedar Rapids, Iowa.

† The constituents used are: Sodium chloride (0.90 percent) 100 ml.; potassium chloride (1.15 percent) 5.0 ml.; calcium chloride (1.22 percent) 1.0 ml.; magnesium sulfate hepta hydrate (3.82 percent) 0.6 ml.; monobasic potassium phosphate (2.11 percent) 1.0 ml.; sodium bicarbonate (1.30 percent) 42.0 ml.; dextrose (12.0 percent) 1.0 ml.; total, 150.6 ml.

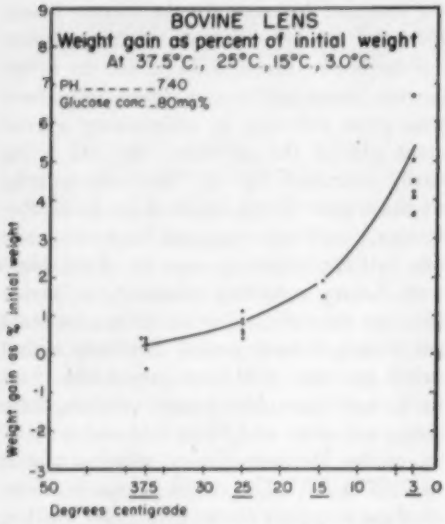

Fig. 1 (Schwartz, Danes, and Leinfelder). Effect of temperature on weight gain of lens.

the rubber sleeve by a hypodermic syringe for pH and chemical determinations. Then the tissue was removed, blotted dry, and weighed.

Blanks (flasks with no tissues present) as well as controls were run in all experiments and the pH determined so as to assure that our experimental variables were not affecting the solutions.

Exposures to different concentrations of oxygen were obtained by bubbling the gas through the solutions containing these tissues —20 minutes for lens and 10 minutes for cornea. The flasks were then sealed.

With this technique we found for the controls: (a) Using generally a 48-hour incubation period for lens, the weight gain was approximately 0.4 percent of the initial weight. The transparency of the lens was also maintained. (b) Using a 24-hour incubation period, the cornea gained 42 percent of its initial weight. The cornea showed peripheral clouding at the limbal area whereas the whole central area was clear and transparent. This increase in weight may be due to injury at the limbus on removal.

Results

Part I. The requirement of metabolic energy to keep water out of the tissue or prevent hydration

The following factors which influence metabolism have been found to affect water and ion uptake of tissues and cells: (1) Temperature, (2) lack of metabolic substrate, (3) anoxia, (4) pH, and (5) enzyme poisons.

The following experiments were performed to determine the role of these factors in lens and corneal hydration:

1. *Temperature.* The metabolism of tissue and thus its energy production will be directly lowered with the lowering of its temperature. Lens and cornea were kept at varying temperatures and their weights measured. As the results show in Figures 1 and 2, there is a gradual increase in weight with the lowering of the temperature. The lowering of metabolism by decreasing the temperature thus results in a directly proportional increase in weight.

2. *Lack of metabolic substrate.* Lens and cornea were placed in solutions in which the

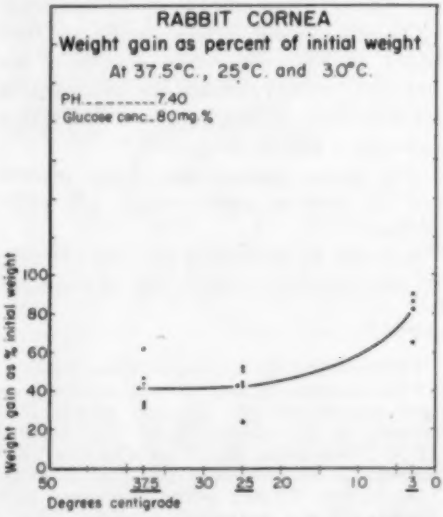

Fig. 2 (Schwartz, Danes, and Leinfelder). Effect of temperature on weight gain of cornea.

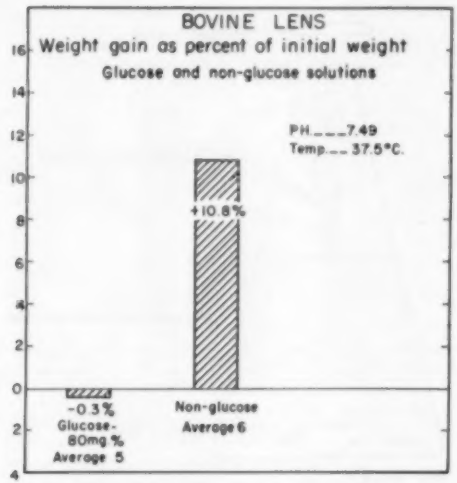

Fig. 3 (Schwartz, Danes, and Leinfelder). Effect of lack of glucose on weight gain of lens.

glucose had been omitted. The tonicity was maintained at the same level as the glucose solutions by increasing the sodium chloride content. As illustrated in Figures 3 and 4, there is a marked increase in weight in the nonglucose solution as compared to the glucose solution. (The weight loss of the control lenses in the glucose solution in this experiment is probably the result of the gain in weight with prolonged lowered temperature during shipment in ice from the abattoir. Then this weight was lost on raising the temperature during incubation.) These experiments indicate that for energy production to maintain weight glucose is necessary as a metabolic substrate.

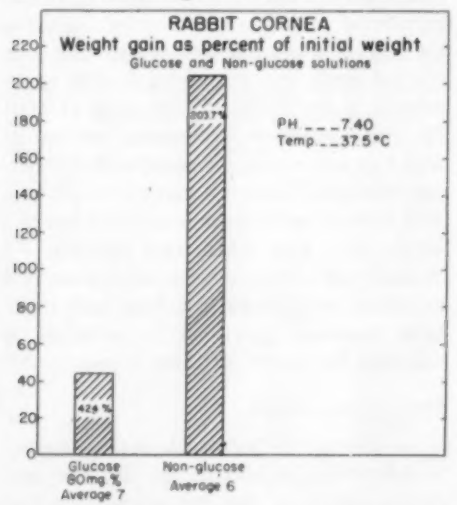

Fig. 4 (Schwartz, Danes, and Leinfelder). Effect of lack of glucose on weight gain of cornea.

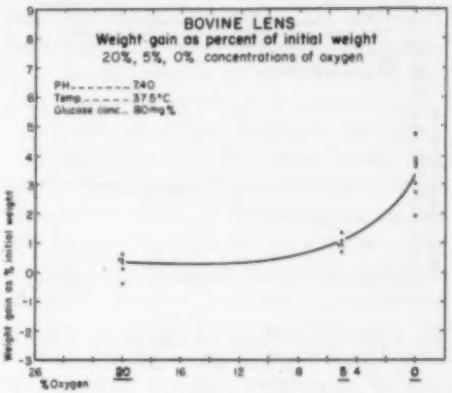

Fig. 5 (Schwartz, Danes, and Leinfelder). Effect of lack of oxygen on weight gain of lens.

3. *Anoxia.* With anoxia there is no available oxygen for respiration. As respiration is a much greater energy producer than glycolysis, lack of oxygen will greatly depress total energy production. Figures 5 and 6 show the effect on weight gain of lens and cornea exposed to varied concentrations of

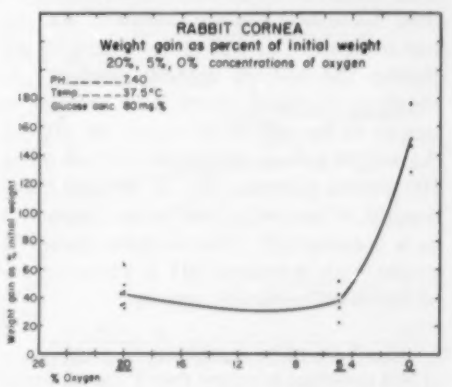

Fig. 6 (Schwartz, Danes, and Leinfelder). Effect of lack of oxygen on weight gain of cornea.

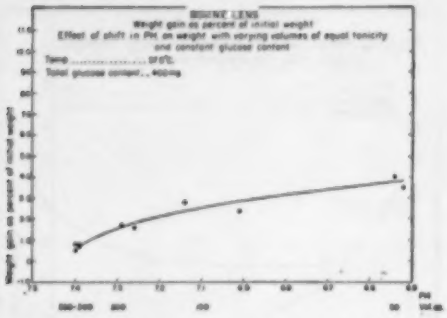

Fig. 7 (Schwartz, Danes, and Leinfelder). Effect of change in pH on weight gain of lens.

oxygen. With both tissues there is very little weight change from 20-percent to five-percent oxygen, but a marked change from five- to zero-percent oxygen or 100-percent nitrogen. Oxygen is thus needed to maintain the weight of lens and cornea. Low concentrations (five percent) are sufficient to maintain weight.

4. *pH.* Bird, et al.[7] have shown that aerobic and anaerobic glycolysis are markedly influenced by changes in pH. Their experiments indicated a linear decrease in the rate of glycolysis with a lowering of the pH. Below pH 7.2 aerobic glycolysis is curtailed one half and anaerobic glycolysis one fifth of their values at pH 7.2 to 7.5. To indicate the effect of pH on weight gain, bovine lenses were placed in decreasing volumes of solution. A constant glucose content of 400 mg. was maintained as well as isotonicity by adjusting the sodium chloride content. As shown in Figure 7, there is an increase in weight as the pH is decreased. At pH 6.5, the weight gain is comparable to that under 100 percent nitrogen (fig. 5). Weight maintenance of lens and cornea is thus dependent on a constant pH. The resultant change in weight with decreased pH is probably due to decreased metabolic activity.*

* Experiments in our laboratory with the growth of lens epithelium in culture show the sensitivity of the tissue to changes in pH of the media. Cessation of growth and death of the culture are soon evident with a decreasing pH.

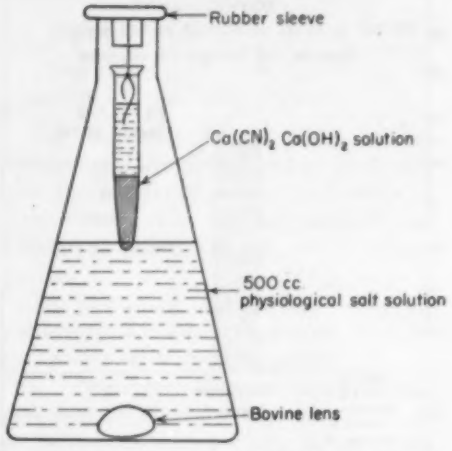

Fig. 8 (Schwartz, Danes, and Leinfelder). Apparatus for maintenance of constant cyanide exposure.

5. *Enzyme poisons.* Cyanide, a cytochrome oxidase inhibitor blocking respiration at the hydrogen transport level, and iodoacetic acid blocking glycolytic activity at the triose phosphate dehydrogenase step were used. Hydrogen cyanide was prepared as described by Robbie[8] so that a final concentration of 0.001 M was obtained. In order to keep this concentration of cyanide constant during the experimental period, a reservoir of $Ca(CN)_2$ $Ca(OH)_2$ was suspended in the flask (fig. 8). Iodoacetic acid was added directly to the solution giving a final concentration of 0.001 M. Figures 9 and 10 illustrate our results with lens and cornea compared with 100-percent nitrogen. There is a marked weight gain with cyanide and iodoacetic acid and less of a weight gain with 100-percent nitrogen. As cyanide and nitrogen block respiration and iodoacetic acid blocks glycolysis both metabolic processes appear to be necessary to maintain the weight of these tissues.

Part I. Conclusions

An attempt has been made in these studies to affect the metabolism of the lens and cornea and show that the weight gain and thus the fluid uptake is directly related to metabolic activity. All solutions used were

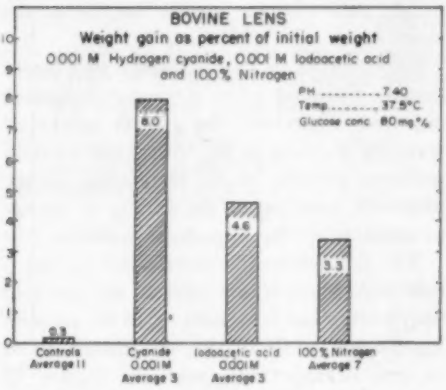

Fig. 9 (Schwartz, Danes, and Leinfelder). Comparison of effect of HCN, iodoacetic acid, and 100-percent nitrogen on weight gain of lens.

isotonic with aqueous in the above experiments. The lens and cornea took up fluid or became hydrated even though the tonicity of these solutions was not changed.

In the maintenance of lens and corneal hydration, our results indicate:

1. The importance of an adequate metabolism as shown by the increase in weight with decreased temperature.

2. The necessity of glucose as a metabolic subtrate.

3. The importance of respiration and the limited capacity of glycolysis as shown by the weight gain under nitrogen and cyanide.

4. The importance of glycolysis as noted with the weight gain with iodoacetic acid.

5. The necessity for a constancy of pH.

Weight gain and thus fluid uptake are not merely a matter of osmotic equilibria but the metabolism of the tissue plays an active role in maintaining a steady state of water content. Only in tissue whose metabolism is decreased or when the tissue is dead would osmotic equilibria alone be important. The concept that both respiration and glycolysis are important energy producers in the metabolism of the lens and cornea is further confirmed. The energy is used to maintain the constant water content and thus the transparency.

Part II. The requirement of metabolic energy to keep water in the tissue or prevent dehydration

The requirement of energy to maintain a constant water content and thus to combat the tendency for water withdrawal by a hypertonic or dehydrating force has been investigated in the following experiments.

Lenses which had been incubated for 48 hours in increasing concentrations of glucose showed a weight loss as shown in Figure 11.

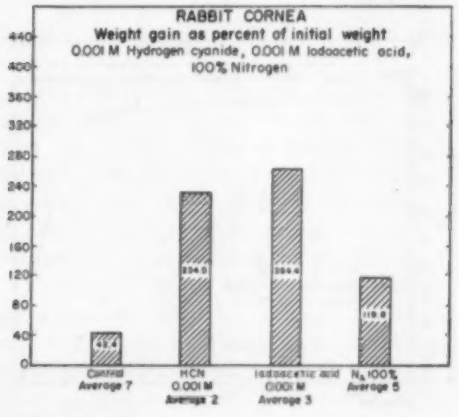

Fig. 10 (Schwartz, Danes, and Leinfelder). Comparison of effect of HCN, iodoacetic acid, and 100-percent nitrogen on weight gain of cornea.

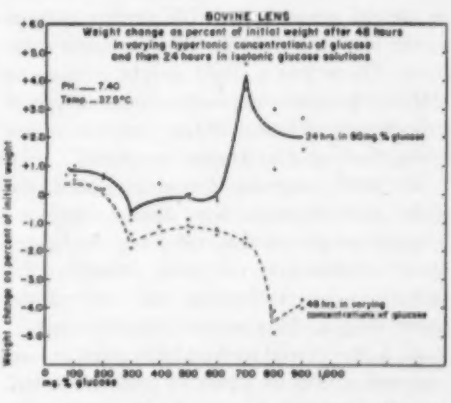
BOVINE LENS
Weight change as percent of initial weight after 48 hours in varying hypertonic concentrations of glucose and then 24 hours in isotonic glucose solutions
PH ... 7.40
Temp ... 37.5°C
24 hrs. in 80 mg % glucose
48 hrs. in varying concentrations of glucose
Weight change as percent of initial weight
mg % glucose

Fig. 11 (Schwartz, Danes, and Leinfelder). Weight-change curves of lenses placed in hypertonic and then isotonic solutions.

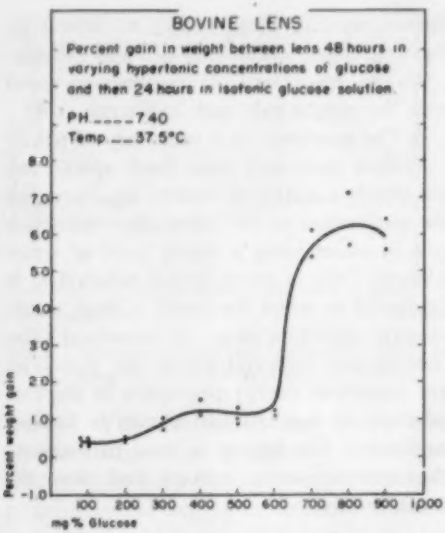

Fig. 12 (Schwartz, Danes, and Leinfelder). Difference between hypertonic and isotonic curves shown in Figure 11.

The loss of weight was small until a 300 mg. percent concentration was reached, after which there was a constant loss until 700 mg. percent. A marked loss of weight is evident after 700 mg. percent.

When these same lenses which had been incubated in hypertonic solutions were then placed in isotonic solutions for 24 hours, they then showed a weight gain. The gain in weight again followed a similar pattern as the loss in weight in the hypertonic solutions. There was a slight weight gain up to 300 mg. percent—a greater constant weight gain between 300 and 700 mg. percent—after which there is a large gain in weight.

If purely osmotic forces regulated the water shift then the lens should regain its original weight on removal from the hypertonic solution and on being placed in the isotonic solution. Instead the lens gained more weight. This was especially noted in those lenses placed in the higher glucose concentrations. Figure 12 which plots the difference between the hypertonic and isotonic curves in Figure 11—that is, the total shift in weight—illustrates clearly the greater weight gain with the greater tonicity of the solution.

Experiments in Part I showed that lenses became hydrated when their metabolism is impaired. Therefore, the greater uptake of water by the lens in the hypertonic solution indicates a stress on the metabolism of the lens with lowering of the facility of energy production by the hypertonic solution.

The dependence on metabolism to maintain a constant water content and prevent dehydration can be shown again by subjecting the lens to decreasing concentrations of oxygen in hypertonic solution. Figure 13 shows the great loss of weight with the lowering of the oxygen tension. With anoxia, no oxygen is available for respiration and the metabolic energy produced by glycolysis is insufficient to prevent dehydration.

Part III. The requirement of metabolic energy for the removal of water or dehydration of the tissue once it has become hydrated

1. *The reversibility of hydration produced by anoxia.* In these experiments an attempt was made to evaluate the ability of the lens and cornea to remove water after being subjected to periods of anoxia.

Lenses were exposed to 24-, 48-, and 72-hour periods of 100-percent nitrogen and

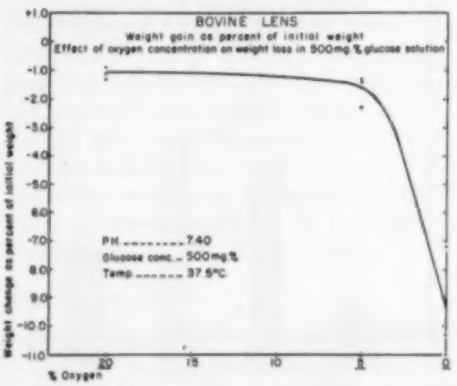

Fig. 13 (Schwartz, Danes, and Leinfelder). Effect of lack of oxygen on weight change of lens in hypertonic solution.

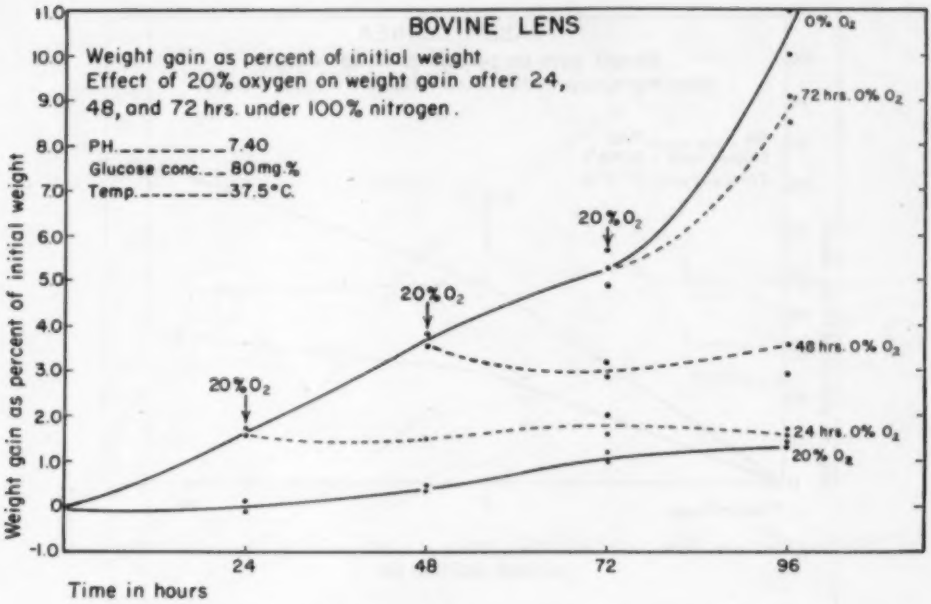

Fig. 14 (Schwartz, Danes, and Leinfelder). Effect on lenses of exposure to 100-percent nitrogen and then air.

then exposed to air. The results are shown in Figure 14. Lenses subjected only 24 hours to nitrogen required about 72 hours to reach the weight level of those lenses in air. With 48-hour exposure to nitrogen the lens just maintained its own weight. Seventy-two hours of nitrogen resulted in a continued weight gain comparable to those lenses in nitrogen 96 hours.

These experiments show that the lens can remove water once it has become hydrated under anoxia. The ability to remove water, however, depends on the respiratory facilities of the lens. After 72 hours of anoxia, the metabolic energy of respiration evidently does not survive to maintain the weight.

Similarly, as shown in Figure 15, rabbit cornea was subjected to 12 hours of 100-percent nitrogen, then exposed to air. The weight gain under anoxia is halted with the return of respiration on exposure to air. There appears to be little if any indication for reversibility in the 12-hour period following exposure. Loss of weight of the cornea may have been more evident if the experiment was carried for a longer period of time.

2. *The reversibility of hydration produced by lowered temperature.* Lenses were hydrated by placing them at temperatures of 3°C. and 15°C. for 24 hours. They were then placed at 37.5°C. for 24 hours and the weights measured. Results are illustrated in Figure 16.

With the increase in temperature the metabolism is raised and thus the energy production. The water taken in at the lowered temperature is removed so that by 48 hours the weights of the lenses have approached the weight level of the controls. By lowering the temperature there is no insult to the respiratory metabolic processes.

The efficiency of both respiration and glycolysis in removing water from the lens in a short period of time can be compared to the previous experiment where water was removed slowly after the lens was subjected to anoxia. Again this experiment illustrates

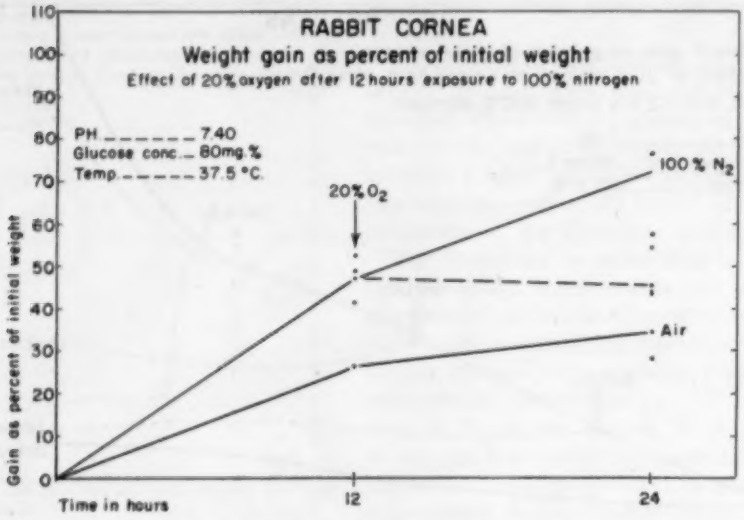

Fig. 15 (Schwartz, Danes, and Leinfelder). Effect on rabbit cornea of exposure to 100-percent nitrogen and then air.

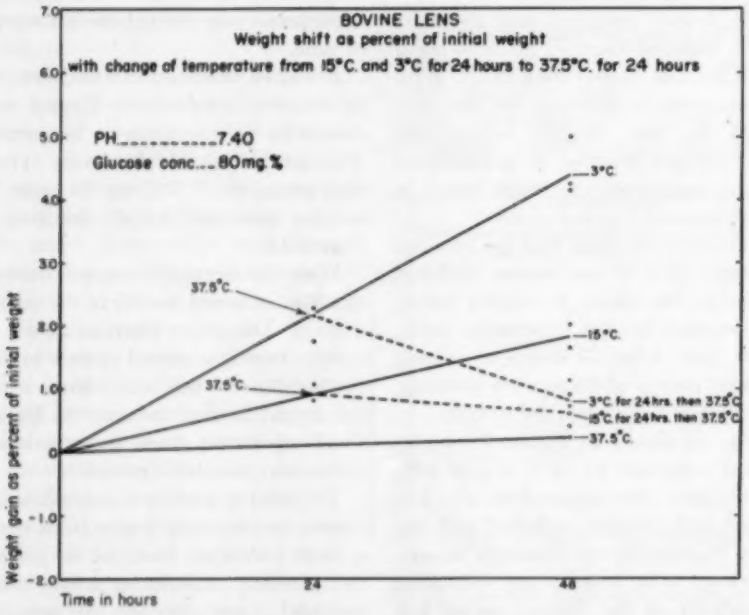

Fig. 16 (Schwartz, Danes, and Leinfelder). Reversibility of lowered temperature hydration of lens.

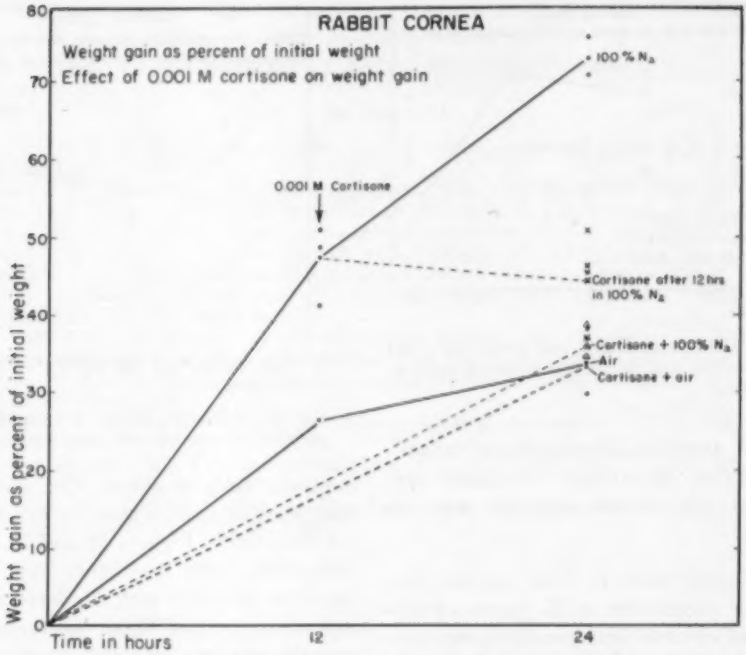

Fig. 17 (Schwartz, Danes, and Leinfelder). Reversibility of anoxic hydration of cornea by cortisone.

the dependency on metabolic energy for the removal of fluid.

3. *The reversibility of anoxic hydration of cornea by cortisone.* The identification of an adrenal cortical factor with the ability of tissues to transport ions and water is indicated in the experiments of Thatcher and Hartman.[10] The reversibility of corneal edema with cortisone and the regression of vascularization is a well established clinical observation. It has been recently debated whether the action of cortisone is one of direct effect on the capillaries,[11] or one of direct action on the cornea itself.[12]

The role of cortisone in reversing the anoxic hydration of the isolated cornea was studied by placing 0.001 M cortisone acetate* in solution with cornea under air for 24 hours, under nitrogen for 24 hours, and under nitrogen for 12 hours, after 12 hours' exposure to nitrogen. Figure 17 illustrates the results which show cortisone exerts no effect on weight gain under air. But under nitrogen, the weight gain of the cornea was almost similar to the controls. Cortisone stopped any further weight gain on addition to cornea after 12 hours 'exposure to nitrogen.

Thus cortisone prevents isolated cornea from hydrating under anaerobic conditions. No definite reversibility was obtained but this may have been evident if the experiments were carried longer. Cortisone thus acts on the cornea itself, raising the metabolic production of energy in some way to prevent hydration. Anoxia seems to be the stimulus. Corneal vascularization probably serves the purpose of providing oxygen and metabolic substrates for energy production. When this demand is met by cortisone vascular regression follows.

* Courtesy of Merck & Co., Rahway, New Jersey.

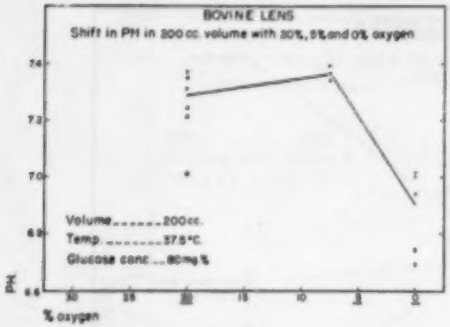

Fig. 18 (Schwartz, Danes, and Leinfelder). pH shift of solution with decreasing oxygen concentrations for lens.

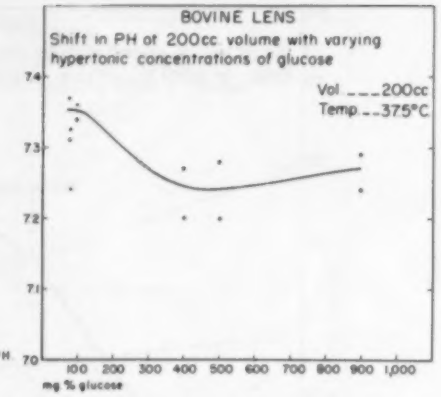

Fig. 20 (Schwartz, Danes, and Leinfelder). pH shift of solution with increasing tonicity.

Part IV. Interrelationships of Energy-Producing Metabolic Processes Required for Water-Content Maintenance

The Pasteur effect is classically described in muscle metabolism as an increased glycolytic activity and thus greater energy production with an anoxic stimulus. To show that similar mechanisms exist in lens and cornea the following experiments have been performed.

Lenses were placed in 200-cc. and cornea in 20-cc. volumes under varying oxygen concentrations so that the buffer capacity of the solution would be easily overcome by any metabolic acids produced. The shift in pH was taken as an indication of increased glycolytic activity. Figures 18 and 19 illustrate our results with the cornea and lens and show the pH shift with the decreasing oxygen concentration. These observations indicate the existence of a Pasteur effect in lens and cornea.

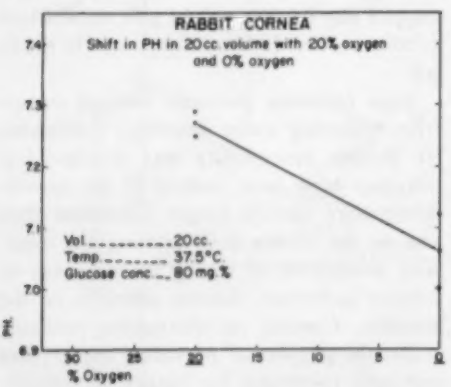

Fig. 19 (Schwartz, Danes, and Leinfelder). pH shift of solution with lack of oxygen for cornea.

By increasing the glucose content of 200-cc. volumes of solution increasing tonicity was obtained. When lenses were placed in such solutions a similar shift in pH was observed as the hypertonicity increased (fig. 20). This hypertonicity exerts a Pasteur-like effect on lens metabolism. The shift to a greater energy production through glycolysis may provide energy to resist the hypertonic force and maintain the water content.

Summary

The maintenance of a constant water content in lens and cornea is an energy consuming process dependent upon the metabolic activity of the tissues. Energy is required to prevent hydration and dehydration of the tissue. Energy is also needed for the removal of water once it has entered as a result of defective metabolism. The lens and cornea can shift their metabolism to an increased glycolytic activity under anoxic and hypertonic stimuli to obtain more energy for

maintaining water content. Since the shift in water content is one of the important processes in corneal and lens pathology, a decrease in the metabolic production of energy is thus indicated.

University Hospitals.

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Discussion

Dr. Albert M. Potts (Cleveland): The work presented by Dr. Schwartz is both interesting and informative, particularly so since the necessity for energy expenditure by the cornea itself is another method of differentiating between the passive dehydration by hypertonic aqueous and tears and an active dehydration by the cornea itself.

In the work we did we got no change with cyanide and iodoacetic acid as we used it. Application of cyanide and iodoacetic acid to the living animal in concentrations adequate to cause effects but not to kill the living animal is a considerably more difficult procedure. Nevertheless, when these materials were injected in reasonably high molarity with our radioactive isotopes, or when they were applied either to the denuded or intact surface of the eye for as long as 10 minutes before our experiment, there was no significant change in hydration nor in corneal transparency that we could note.

On the other hand, under these circumstances it is quite possible that there was no significant effect on the metabolism of the cells in which we were particularly interested.

I would like to ask the author whether he has independent evidence for the increase in metabolic activity caused by application of cortisone as he used it.

Another question that occurs to me is whether, over this period of time, under the rather damaging conditions that were used, the actual cellular integrity of corneal epithelium and endothelium and of cell capsule were maintained. This does not in any way reflect upon the results produced but, if there were a physical break in the membrane at any time, one might expect a different order of magnitude of effect after the break occurred.

Dr. V. Everett Kinsey (Detroit): The conclusions drawn from the experiments, however, with regard to the dependence of the maintenance of water balance in the lens and cornea upon respiration leave me somewhat confused when I recall the conclusions arrived at by Dr. Leinfelder and his group from other experiments which were reported last year. If I remember correctly, one of the group presented a paper in which the authors concluded that the oxygen uptake of the lens is nonenzymatic, and, hence, by inference, that the lens does not respire. I would appreciate some comment by Dr. Leinfelder on this apparent disparity of view.

It seems to me dangerous to conclude that the operation of the Pasteur effect leads to an increase in energy production. If I recall correctly, the essayists said that more acids were produced with decreasing concentrations of oxygen. It was concluded that this increase in anaerobic glycolysis would be accompanied by greater energy production. If the quantity of lactic acid produced increased, as the results seemed to indicate, the total energy available to the lens, I believe, might well decrease rather than increase, as stated by the authors, for it is well known that the energy produced per mole of glucose consumed through burning of the acids (chiefly pyruvic acid) is many times that which results merely from the glycolytic breakdown of glucose.

Dr. P. J. Leinfelder: I certainly do want to comment regarding Dr. Kinsey's question, because it is diametrically opposed to what we believe. This whole question was explained in a paper given at

the American Ophthalmological Society this year, and it will be published in the transactions of that group.

However, in summary, our experiments, reported two years ago on boiled lenses, indicated that the oxygen uptake, as measured in the Warburg apparatus, was perhaps unreliable because it was not an index of respiratory activity of the tissue and, in the same paper, we reported that the estimation of glycolysis by the Warburg technique is a valid measurement because it is a heat-stable mechanism.

It was our contention—perhaps not expressed in that paper, but expressed in the paper which was given at the American Ophthalmological Society—that the respiratory processes in the lens are limited to the epithelium, and that the amount of respiration in the epithelium is so slight that it is overshadowed by the autoxidation of glutathione and ascorbic acid which is registered when you measure the oxygen uptake of the entire lens.

In regard to the cyanide experiments, Dr. Potts, we have found that, even under the best circumstances in vitro, 20 minutes are required to establish equilibrium, and it is no wonder that tissue response was not available.

In our earlier experiments, when Dr. Robbie was still alive, we did numerous experiments in injecting cyanide in the eye, and we were always disappointed in not being able to get an effect.

EXPERIMENTAL GRANULOMATOUS UVEITIS*

STUDIES ON THE MECHANISM OF PRODUCTION OF THE CONTRALATERAL REACTION AFTER THE USE OF HORSE SERUM IN RABBITS

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(With the technical assistance of Jack M. Tadman, M.S.)

Indianapolis, Indiana

Several authors have made contributions to the study of experimental horse serum-induced granulomatous uveitis in rabbits.[1-12] Schlaegel showed that a granulomatous uveitis with a histopathologic picture resembling that of sympathetic ophthalmia could be produced in both eyes of a rabbit by an injection of horse serum into one eye followed in three weeks by horse serum intravenously.[5] This procedure provides an unusual opportunity for antigen-antibody determinations in the contralateral or "sympathizing" eye, uncomplicated by previous manipulations. It is the purpose of this study to investigate some of the mechanisms which may be responsible for this form of contralateral granulomatous uveitis.

The following major facts have been established:

1. If the eye of a rabbit is injected with horse serum, a granulomatous uveitis will develop in the injected eye at about 14 days.

2. If the rabbit has been sensitized previously by intradermal injections of horse serum, uveitis will develop within one day instead of 14 but the contralateral eye remains uninvolved.

3. After the injection of horse serum into one eye of a normal rabbit, if horse serum is given intravenously one to three weeks later, uveitis will develop in the contralateral untreated eye.[4]

4. The degree of contralateral uveitis is increased by use of the eye instead of the skin as a site of sensitization and also is increased by the use of large amounts of horse serum intravenously (for example, 20 ml.).[5]

In the experiments being reported, two possible mechanisms were considered: (1) A direct toxic effect of horse serum on the uvea and (2) an allergic reaction involving either (a) local or (b) systemic antibodies.

EXPERIMENT 1

POSSIBLE PRODUCTION OF UVEITIS BY DIRECT TOXIC ACTION OF HORSE SERUM

Method. Each of 15 rabbits received 20 ml. of horse serum intravenously. The rabbits were killed at four through 21 days and their eyes enucleated. Eighteen scattered

* From the Department of Ophthalmology, Indiana University School of Medicine. This work was supported by a grant from the National Council to Combat Blindness, Inc.

sections from each eye were cut, stained with hematoxylin and eosin, and graded as to the degree of cellular reaction. The grading of sections in this and other experiments being reported was made by one of us (T.F.S.) without knowledge of their source. These gradings were from 1 through 4, based on the degree of cellular infiltration.

Results and Interpretation. Figure 1 demonstrates that no uveitis appeared during the first week. Uveitis began to appear during the second week and, although minimal in degree, reached a peak at 16 days. Since an antigen-antibody reaction usually begins some time after seven days and reaches a height at about 14 days, the development of uveitis within the first week would have indicated that horse serum produces a uveitis by direct toxic action. Since the uveitis was delayed until the classic period for an antigen-antibody reaction, the results support the antigen-antibody postulate.

Three experiments to differentiate between local and systemic antibody mechanisms

Experiment 2

Method. Each of 25 rabbits received 0.1 ml. of horse serum in the right eye. Three weeks later each rabbit was injected intravenously with 20 ml. of horse serum.

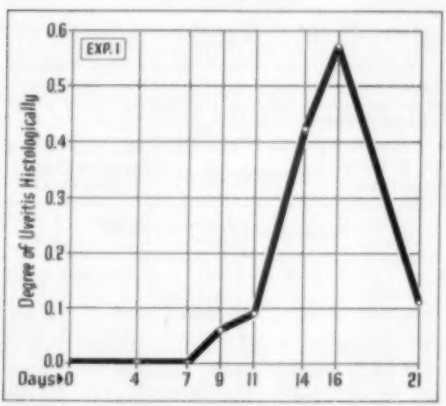

Fig. 1 (Schlaegel and Wilson). The degree of histopathologic uveitis at the days indicated after the intravenous injection of 20 ml. of horse serum.

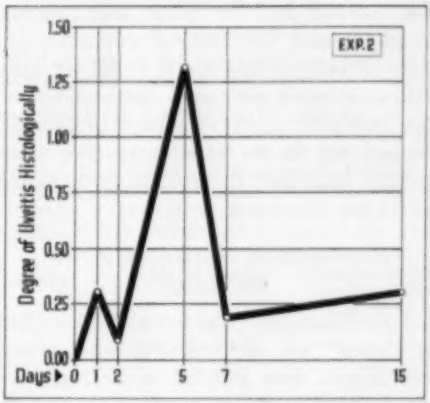

Fig. 2 (Schlaegel and Wilson). The degree of histopathologic uveitis in the untreated left eyes at the days indicated. These rabbits had received an injection of horse serum in their right eyes followed by 20 ml. intravenously in three weeks.

Results and Interpretation. Figure 2 demonstrates that uveitis was present within one day and reached a maximum development after five days following the intravenous injection of horse serum. It is possible, but unlikely, that any of the horse serum injected into the right eye would reach the left eye to stimulate the production of local antibodies. Uveitis developed in the first week after intravenous horse serum, so the postulate of systemic antibodies is supported; if the uveitis had been produced by local antibody reaction it should have been more delayed.

Experiment 3

In this experiment the titer of antigen and antibody was measured in both blood serum and aqueous humor.

Method. The right eyes of 30 rabbits were injected with 0.1 ml. of horse serum. At three weeks, 20 ml. of horse serum was given intravenously. Blood and aqueous samples were collected at intervals before and after this intravenous injection of horse serum. Six rabbits were used for each

time interval and the blood and aqueous samples tested for antigen and antibody. Since secondary aqueous is richer in antibodies, a rabbit was used only twice and then only after a rest of at least two weeks. Because fat in the blood may give false positive reactions, the rabbits were fasted before the samples were drawn.

Determination of antihorse-serum antibodies

A modification of the technique described by Hanks[13] was adopted. The horse serum was diluted with distilled water and then incorporated in the gelatin base to give the desired final concentrations. The gelatin base is composed as follows:

Gelatin (Difco)	5.0 gm.
NaCl	1.0 gm.
Phenol	0.5 gm.
Glycerol	20.0 ml.
Distilled water	80.0 ml.

After the ingredients are dissolved the pH is adjusted to 7.0 with N/10 NaOH.

The gelatinized antigen is liquefied in a 37°C. water bath and pipetted into the bottom of 6.0 by 50 mm. precipitin tubes, after which it is chilled in the refrigerator until solid. The aqueous humor is then layered over the antigen.

The tests are incubated for 48 hours at 20°C. At the end of this period the tubes are examined for a precipitate at the interphase of antigen and aqueous humor.

Besides the titrations on the aqueous humor, serum from the rabbits used in these experiments was also titrated for the presence of antihorse-serum antibody. The gelatinized antigen test also was used for these tests.

Determination of horse serum in aqueous humor

The ring test was used to detect the presence of horse serum in the aqueous humor. Two-tenths ml. of pooled antihorse serum (rabbit serum, ring test titer—1:6,400) was placed in the bottoms of 6.0 by 50 mm. precipitin tubes. The diluted aqueous humor, or test serum, was then stratified over the antiserum and the tubes incubated at room temperature for four hours. The reactions were noted by holding the tubes before a source of obliquely transmitted light.

Results (Table 1) and Interpretation.

1. After the injection of 0.1 ml. of horse serum into the right eyes, none was detected in the blood or aqueous of the contralateral eye *at the days tested,* presumably because of the small amount injected.

2. The injection into the right eyes produced an appreciable antibody titer in the blood; some of these antibodies entered the contralateral eyes after some lag.

3. Three days after the intravenous injection, the antibody titer in the blood had fallen drastically and antibodies in the aqueous were no longer detectable. The intravenous antigen apparently removed the antibodies by combining with them.

Experiment 3 supports the postulate of systemic antibodies.

Experiment 4

Method. Fifteen rabbits received 10 ml. of rabbit serum intravenously; this serum

TABLE 1

Experiment 3

(The right eyes of 30 rabbits were injected with 0.1 ml. of horse serum. An intravenous injection of 20 ml. was given at 21 days. At the days indicated the left aqueous and blood of six rabbits were tested for antigen and antibody. The mean of these values appears in this table.)

No. of Days from Injection into Right Eyes	Blood Serum		Aqueous Humor (Left eyes)	
	Antigen	Antibody	Antigen	Antibody
7	0*	1/132	0†	1/1.66
10	0	1/164	0	1/3.33
14	0	1/4800	0†	1/6.66
17	0	1/4660	0	1/11.6
I.V.—21				
24	1/800	1/25	1/4.5	0
27	1/203	1/3466	0	1/1.66
35	1/7.5	1/9280	0†	1/3.3

* Two of five rabbits had a 1:2 titer.
† One of six rabbits had a 1:2 titer.

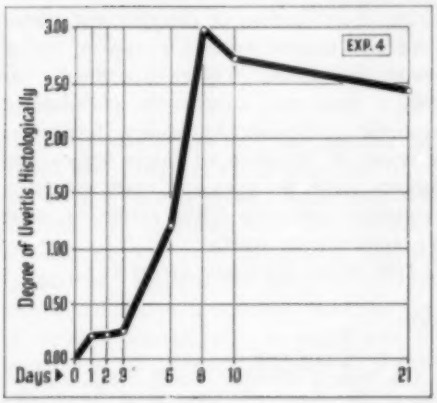

Fig. 3 (Schlaegel and Wilson). The degree of histopathologic uvetitis at the days indicated. These rabbits had received antihorse serum antibodies intravenously followed on the next day by an injection of the antigen (0.1 ml. of horse serum) into the vitreous.

was from rabbits made highly immune to horse serum. On the next day both eyes were injected with 0.1 ml. of horse serum.

Results and Interpretation. Figure 3 demonstrates that uveitis was detected at one day and reached a peak on the eighth day. According to the systemic antibody postulate, antibodies reach the contralateral eye first and react with subsequently appearing horse serum. In this experiment we simulated these conditions by injecting antibodies intravenously and by supplying the antigen by injection into the eyes on the next day. Since the uveitis which was produced was severe and in every way comparable to that previously produced,[1, 2] the postulate of systemic antibodies is supported.

Elective sensitization

Experiment 5

Method. One group of 15 rabbits was sensitized to horse serum by an injection into their *right* eyes and another group of 15 rabbits by an injection into the skin of the back. Skin and *left*-eye sensitivity tests were done on both groups after two weeks. The skin and *left*-eye reactions were graded on a 0 through 4 scale. Then the left eyes were enucleated, studied for the degree of uveitis, and graded on a 0 through 4 scale.

TABLE 2

Experiment 5

(Fifteen rabbits of a *skin group* were sensitized to horse serum by an injection into the skin of the back. Another 15 rabbits, comprising an *eye group*, were sensitized by an injection into the right eye. At two weeks, left eye and skin sensitivity tests were performed on both groups and the left eyes later sectioned and graded for degree of uveitis. There is no significant difference between the skin and eye groups.)

Group	Degree of Clinical Reaction		Degree of Histopathologic Uveitis in the Left Eyes
	Left Eye	Skin	
Skin	2.7	2.2	2.49
Left Eye	3.1	2.6	2.65

Results and Interpretation. Table 2 demonstrates no significant difference between the skin and eye groups after calculation of T. It is possible that an injection into the right eye of a rabbit could in some way especially predispose it to an ocular reaction on the contralateral side simply because the sensitizing injection was made into an eye and not elsewhere. If elective sensitization had occurred, the eyes of the eye group and the skin of the skin group should have shown the greater reactions. There is a tendency for the eye group to give higher readings in all three categories. These findings are against elective sensitization and evidence only of the possibility that eye injections will produce a greater general sensitivity than will injections into the skin of the back.

Comment

The body of evidence now accumulated strongly supports the role of systemic antibodies in the production of uveitis from horse serum although it does not rule out the possibility that local antibodies also play a part. The evidence does not prove that the combination of antigen and antibody in itself stimulates the granulomatous response.

Neither does it mean that histamine which might be liberated by the antigen-antibody reaction is the agent, since histamine has been proved incapable of inciting cellular infiltration.[14] What substance stimulates the granulomatous response remains unknown.

Summary

Five experiments were performed on 100 rabbits in an attempt to elucidate the mechanism of production of contralateral granulomatous uveitis from the use of horse serum in rabbits. These experiments, as well as past ones, support the postulate of systemic antibodies. Antibodies, formed as a result of injection of horse serum into rabbits, enter the eyes and react with subsequently appearing horse serum to incite a granulomatous uveitis.

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TREATMENT OF EXPERIMENTAL OCULAR TUBERCULOSIS WITH ISONIAZID*

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It was planned to find out whether isoniazid would prevent the development of ocular tuberculosis if given concurrently with the infecting inoculum, and how it would affect the course once the disease was well established. To test the drug under most severe conditions, a massive infective dose of virulent human tubercle bacilli was injected into the anterior chamber of normal rabbits since the reports of Woods[1] and Bietti[2] have shown that normal rabbits offer a more rigid test of the efficacy of an antitubercular drug than do immune-allergic rabbits. As the tubercle bacillus is capable of lying dormant for months and then becoming active again, it was necessary to observe the rabbits for as long a period as feasible after treatment was completed in order to see if there were clinical recurrences or if viable tubercle bacilli remained in the eyes of the treated animals.

EXPERIMENTAL CONDITIONS

In order to find a suitable infective dosage three range-finding experiments utilizing 36 eyes of 18 rabbits were performed. It was determined that 0.05 cc. containing 50,000± viable virulent tubercle bacilli of a seven- to 10-day culture of H_{37} Ru strain grown on Trudeau commercial egg media when introduced into the anterior chamber of a normal rabbit produced a uniformly severe ocular tuberculosis. Dosage was determined by serial dilutions of a suspension of 0.1 mg. dry weight of the culture per cc. according to the technique of Bunn and Brabeck.[3]

In order to minimize leakage the injections into the anterior chamber were made with a 30-gauge needle on a tuberculin syringe penetrating the sclera about four mm. behind the limbus and passing through the ciliary body and iris so that these structures would act as a stopper. This route appeared to be relatively atraumatic as no marked signs of traumatic iridocyclitis developed and in only one case did a small anterior lens opacity occur.

This method was evolved because the previously employed technique of introducing a 30-gauge needle through an oblique intracorneal route did not prevent gross leakage. These details are stressed as it is felt that this procedure would insure a relatively uniform infection dose.

On March 17, 1952, 100 eyes of 75 apparently healthy normal adult rabbits of 2,700-gm. average weight were inoculated with the above-mentioned dosage and technique. These rabbits were divided into the following groups:

TREATMENT	mg./kilo	RABBITS	EYES
Immediate	10	15	20
Immediate	25	15	20
Delayed	10	15	20
Delayed	25	16	22
Controls	—	14	18

Immediate treatment began on the same day as the infective inoculum was injected. Delayed treatment was started two weeks later. Half of all of the treated rabbits received subcutaneously 10 mg. per kilo of isoniazid (Rimifon®)† daily in solution of 40 mg. per cc. of sterile distilled water. The other half was treated with 25 mg. per kilo. As the higher dosage showed no therapeutic advantage over the lower dose, and as it appeared to be toxic, all rabbits received the 10 mg. per kilo dosage from the eighth

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† Rimifon®, Hoffman-LaRoche, Inc.

week through the 24th week when treatment was stopped. Thus there remained three groups: Control, immediate treatment, and delayed treatment.

Every rabbit was examined twice a week for eight weeks and weekly thereafter. The amount of inflammatory reaction was graded as follows:

0 = No signs of active inflammation though scarring may be present.
+ = Keratic precipitates, slight hyperemia of the iris, one to four iris nodules, or minimal exudate.
++ = Over four iris nodules, more marked hyperemia, and more marked exudate.
+++ = Corneal haze, vascularization with beginning abscess formation, moderately severe iris involvement.
++++ = Full-blown conglomerate tuberculosis of the anterior segment.

At the time of killing the rabbits, each eye was either fixed in Zenker's solution for embedding in celloidin and histologic study, or the anterior uvea was removed under aseptic conditions for bacteriologic examinations. Ten sections of each eye were stained with hematoxylin-eosin and five sections with a modified Ziehl-Neelsen technique to study changes in the tissues and to detect the presence of acid-fast organisms.

The results of histologic examination were graded according to:

0 = No more than occasional round cells in anterior uvea and at limbus. Evidence of previous scarring discounted.
+ = Minimal but definite round-cell infiltration in anterior uvea and at limbus.
++ = Moderate round-cell infiltration of the same regions.
+++ = Marked round-cell infiltration with tubercle formation of the same regions.
++++ = Conglomerate tuberculosis with caseation of the globe.

The aseptically removed material was used for direct smears and culture. In addition, part of the tissue was ground up and suspended in physiologic saline solution for injection into the groin of a guinea pig. This suspension was also concentrated by the sodium hydroxide method for examination by smear, culture, and guinea-pig inoculation.

Results

I. Toxicity

There were 23 deaths in this series. As 18 rabbits appeared too ill to survive, they were killed so that the eyes could be studied without postmortem changes. Five rabbits died spontaneously. Of these 23, one had received no treatment and one belonged to the control group. Of the remaining 21, six had been receiving 10 mg. per kilo of isoniazid daily, whereas 15 had been on the higher dosage schedule. The high death rate in the latter group suggested a toxic side effect of isoniazid, particularly since all fatalities occurred during the first six weeks of treatment.

Two weeks after all of the rabbits had been placed on the lower dosage schedule not a single experimental animal succumbed. Moreover, the rabbits on the higher dosage schedule had been the only ones who failed to gain weight, whereas after the dosage was reduced, all of the rabbits gained weight, averaging 1,000 gm. per rabbit over the treatment period of five and one-half months.

II. Clinical course

The clinical course of the three groups during the period of treatment is shown in graphic form on Figure 1. As indicated on the graph, all rabbits developed conjunctival injection, iris hyperemia, and slight exudate near the pupil on the second to fourth day.

In the immediately treated group the inflammatory signs had subsided in all but two eyes by the second week, so that treatment was stopped. In the third and fourth weeks thereafter all but five eyes showed a recurrence of the tuberculous process, so that treatment was restarted.

All of these rabbits responded to therapy dramatically, except one in which the infection progressed to advanced ocular tubercu-

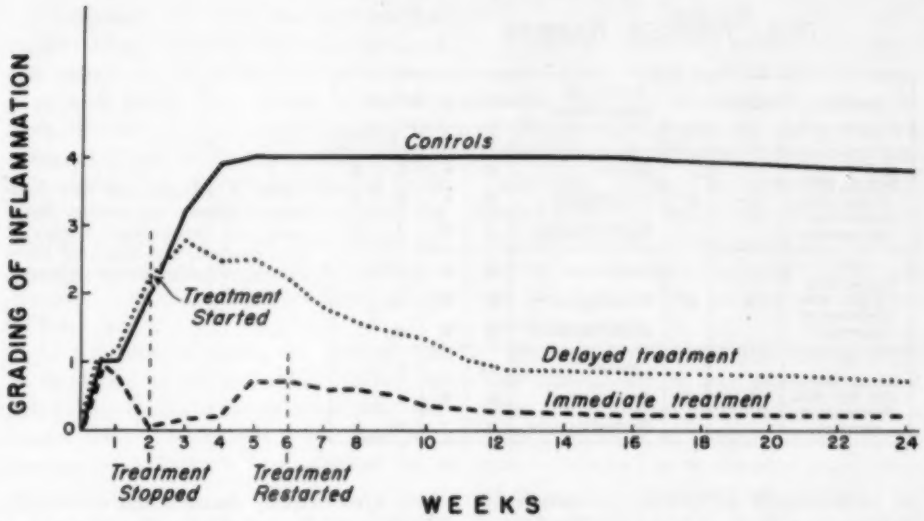

Fig. 1 (Knapp and von Sallmann). Effect of immediate and delayed treatment with isoniazid on the clinical course of experimental ocular tuberculosis in normal rabbits.

tosis. This animal died spontaneously four weeks after treatment had been restarted. This was the only indication of the development of drug-resistant organisms in this series.

At the end of treatment all but five eyes were normal clinically. These five showed minimal exudate, keratic precipitates, or iris nodules. The other 24 were free of signs of active inflammation.

In the control group of 13 rabbits the immediate inflammatory process advanced without exception until at the end of four weeks every eye showed a picture of a massive ocular tuberculosis that continued to progress to actual perforation in three instances; five rabbits had to be killed as perforation appeared imminent. The course of the disease was uniform except in one rabbit. Here the inflammation began to subside in the fourth month. At the time of death in the 10th month this eye was atrophic and scarred but still showed evidence of active tuberculous inflammation on histologic examination.

In the delayed treatment group, all eyes showed definite ocular tuberculosis by the end of the second week at which time treatment was started. This inflammatory reaction increased during the first week of treatment, leveled off for two weeks, and then began to display dramatic improvement so that by the end of the period of treatment only two eyes presented signs of pronounced activity, eight eyes showed minimal or questionable activity, and the remaining 15 eyes appeared entirely free of active inflammation.

III. Results of bacteriologic and histologic examination

At the end of the five and one-half month period of treatment, there remained for further study 10 eyes of eight control rabbits and 54 eyes of 40 treated rabbits (21 immediate treatment and 19 delayed treatment). These animals were killed in three groups as noted in Figure 2.

The first group, killed on September 12, 1952, 13 days after cessation of treatment, consisted of 16 eyes of 11 treated rabbits.

On clinical examination, 10 were free of any inflammatory signs, four showed minimal activity, and two presented the picture

TOTAL TREATED RABBITS

GROUP	RABBITS	EYES	TYPES OF EXAMINATION	GRADING OF RESULTS			
				0	+	++	+++
I Sept. 12, 1952 (12 days after termination of treatment)	11	16	*Clinical*	10	4	0	2
			Histological	5	4	0	1
			Bacteriological	2	3	1	0
II Nov. 14, 1952 (76 days after termination of treatment)	8	12	*Clinical*	6	6	0	
			Histological	2	2	1	
			Bacteriological	7	0	0	
III Jan. 12, 1953 (135 days after termination of treatment)	21	26	*Clinical*	23	1	2	
			Histological	6	9	1	
			Bacteriological	6	3	0	1

Fig. 2 (Knapp and von Sallmann). Results of clinical, histologic, and bacteriologic studies of eyes enucleated at indicated intervals after cessation of treatment.

of experimental tuberculosis, although hyperemia and other signs of acute inflammation were absent.

On bacteriologic examination the one clinically active eye was positive on all types of examination: smear, culture, and animal inoculation. Three eyes gave positive smears and cultures but were unexpectedly negative on guinea-pig inoculation. The remaining two eyes were entirely negative.

On histologic examination, the other eye with the clinical picture of active tuberculosis revealed extensive granulomatous changes of the anterior segment. Four eyes showed moderate round-cell infiltration at the limbus, in the tissues of the filtration angle, and in the anterior uvea. The remaining five eyes were essentially normal except for a few round cells at the limbus and in the anterior part of the uvea.

Two control rabbits with three infected eyes were also killed at this time. The pathologic changes of all three eyes were graded four-plus on clinical, bacteriologic, and histologic examinations.

Eight rabbits with 12 affected eyes were killed on November 14, 1952, two and one-half months after the termination of treatment. There had been no frank reactivation of the tuberculous process, although in three eyes existing changes had moderately progressed but not to the extent of changing the grading. That is, in one eye two minute specks of exudate had increased in size until they were easily visible; in two others the number of iris nodules had increased from one to three. In none of the eyes was hyperemia of the iris or injection of the circumcorneal vessels observed.

On clinical examination, six eyes showed one-plus activity and the remaining six eyes appeared entirely quiescent. Bacteriologically, all seven eyes examined were negative. On histologic examination one eye showed two-plus activity, two eyes showed one-plus inflammation, and two eyes were negative.

At the same time five of the remaining six rabbits of the control group were killed; one eye had actually ruptured and in the other five eyes rupture seemed imminent. Interestingly, these eyes had not quieted down but appeared to become thin-walled distended bags of pus. For this reason, only one eye was examined bacteriologically and was strongly positive on all tests. The remaining five eyes were examined histologically and graded four-plus, although the tuberculous process was even further advanced than this grade indicates; it had invaded the lens and extended extraocularly.

On January 12, 1953, four and one-half months after treatment had been completed, the remaining 21 rabbits with 26 affected eyes were killed. Two definite recurrences were observed in the fourth month. One occurred in an eye that presented evidence of scarring but the other appeared in an eye that seemed clinically to be normal. Injection of the globe, slight haze of the cornea, hyperemia of the iris with exudate in the pupil, and iris nodules developed in both of these eyes.

At the time of death, the clinical signs were graded as of moderate character but from the progress of the inflammation a full-blown ocular tuberculosis might have been anticipated had death been delayed for a longer period.

One eye was examined bacteriologically with positive results on all tests. The histologic examination of the other eye revealed moderate inflammatory changes.

On clinical examination 23 of the remaining 24 eyes were considered normal. The other eye showed one-plus activity. The results of the bacteriologic examination of six eyes were negative and of three eyes weakly positive. On histologic examination six eyes were essentially normal, and nine revealed scattered round cells in the anterior segment.

In addition to the previously mentioned examinations, 50 eyes were stained by the Ziehl-Neelsen method and five sections of each eye were examined under oil immersion for the presence of tubercle bacilli. Organisms were found in 15 eyes. Nine of these positive results belonged to the control group or to rabbits which were killed in the first six weeks of the experiment.

All of these eyes had shown active inflammation clinically. Of the six bacteriologically positive eyes of rabbits that had undergone the full course of treatment, three had shown clinical evidence of active inflammation and the remaining three had revealed extensive scarring but no evidence of active inflammation.

Comment

Under the conditions of this experiment isoniazid was found to be an effective agent for the treatment of tuberculosis. In doses of 10 mg. per kilo per day it was well tolerated; doses of 25 mg. per kilo per day produced toxic effects. The higher dosage appeared to have no therapeutic advantage over the lower dosage. According to the report of Kratka and Leopold,[4] a dosage of 20 mg. per kilo body weight per day was well tolerated in rabbits.

Isoniazid inhibited the development of ocular tuberculosis in our series. However, when the period of treatment was short, a large percentage of animals showed recurrences three to four weeks after termination of therapy. If treatment was continued for a long period, 130 days in this experiment, one out of 12 animals developed a recurrence in the fourth month after treatment was stopped.

Isoniazid produced dramatic improvement in well-established experimental tuberculosis of the anterior segment of the rabbit eye. After 155 days of treatment, only one out of 14 eyes showed a relapse in the fourth month after termination of therapy.

Comparison with other reports on the use of isoniazid in the treatment of ocular tuberculosis is difficult due to differences in species of experimental animal, technique, and duration of treatment and observation. Pannarale and Leonardi[5] used guinea pigs as experimental animals. In nonimmune allergic animals the drug was ineffective: intraocular tuberculosis developed after preventive treatment was interrupted, and therapy did not prevent progression of the tuberculous process.

Fust, Brückner, and Studer[6] infected the eyes of 17 rabbits with bovine tubercle bacilli. One month after infection the rabbits were treated with Rimifon® locally with instillation of a 1.0- to 1.5-percent solution, and subcutaneously in doses of 10 mg. per kilo. Four rabbits died shortly after

treatment was started. The authors attributed this effect to a Herxheimerlike reaction. The eight surviving animals did well; there were no recurrences two and one-half months after treatment was discontinued.

At the fifth annual conference of the staff and alumni of the Wills Eye Hospital, March 20, 1953, Kratka and Leopold,[4] describing experiments in which the infective dose was defined by counts of three to five organisms per oil immersion field, reported satisfactory results with 90 days of treatment. No recurrences were noted in a three-month observation period.

At the meeting of the Residents Association of the Wilmer Institute, March 25, 1953, Becker[7] reported on the use of isoniazid in experimental ocular tuberculosis. Becker and Woods used 72 normal nonimmune rabbits for the inoculation of the anterior chamber with human tubercle bacilli. In the 24 control animals the infection ran the usual fulminating course. The treatment of 24 animals at the time of inoculation prevented development of the inflammation during the six weeks of treatment, but three weeks later the infection flared up, and only six of eight eyes responded to retreatment. In 24 rabbits treatment was instituted after definite lesions had developed. Regression of all signs of disease was noted in all animals after six weeks of treatment.

Although it is not possible to compare our results directly with those reported thus far by other investigators due to the previously mentioned differences in experimental conditions, it is obvious that there is relative agreement on some points. Thus it has been shown that isoniazid can prevent the development of experimental ocular tuberculosis when treatment is continued for a sufficient length of time. This prolongation of therapy is necessary to prevent relapse. Moreover, isoniazid produces dramatic improvement in established ocular tuberculosis which might be lasting if treatment were continued for a long time.

Summary

1. The results of treatment of ocular tuberculosis with isoniazid in 75 normal nonimmune-allergic rabbits are presented. Isoniazid was shown to be an effective antitubercular drug both in preventing and treating experimental ocular tuberculosis.

2. The necessity of the continuation of treatment beyond clinical cure must be emphasized as typical histopathologic changes and tubercle bacilli could be demonstrated in quiescent eyes and two late recurrences were encountered in our series.

630 West 168th Street (32).

We wish to express our appreciation to Dr. Joseph D. Aronson and Dr. Max B. Lurie, of the Henry Phipps Institute, University of Pennsylvania, and Dr. Emanuel Grunberg and Dr. Robert J. Schnitzer, of Hoffmann-LaRoche, Inc., for their helpful suggestions and advice, and to Mrs. Clare Kirpisch for her assistance in part of the experimental work.

We are greatly indebted to Hoffmann-LaRoche, Inc., for supplying us with the preparation, Rimifon®.

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Discussion

Dr. T. F. Schlaegel (Indianapolis): I want to congratulate Dr. Knapp and Dr. von Sallmann on this excellent piece of work.

I would like to ask why such a large dosage was used. The usual clinical dosage is three to five mg. per kilogram. An interesting recent report indicates that larger doses are not necessary nor advantageous, because isoniazid penetrates easily into a cell, and therefore increasing the dosage has no greater effect. That came out, of course, after their work was practically finished.

Dr. Philip Knapp (New York): At the time this experimental study was started, that is a year and a half ago, less was known about dosage of isoniazid than is known now. At that time the available reports on experimental work referred to 10 mg. per kilogram of the compound. We were interested in determining the toxic level, and therefore used the higher dosage.

Interestingly enough, I think Leopold and Kratka employed in rabbit experiments 20 mg. per kilogram per day. They did not observe toxic effects with that dose. It seems then that, for the rabbit, the toxic level lies between 20 and 25 mg. per kilogram.

HEALING OF THE POSTERIOR LENS CAPSULE AND LENS-INDUCED ENDOPHTHALMITIS*

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(With the technical assistance of Martha C. Harrison)

New Orleans, Louisiana

Our own previous studies on lens-induced endophthalmitis,[1] as well as reports in the recent literature, have interested us in the possible relationship of the posterior lens and its capsule to this disease. The experimental study to be reported in this communication was undertaken to supplement the existing clinical knowledge and to determine (1) if there is a mechanism of posterior lens healing after trauma or disease, and (2) the response of the posterior ocular segment to extruded lens material. Finally, an attempt has also been made to correlate the experimental findings in this study with the findings in human eyes.

Although posterior proliferation of lens epithelium has been described by a number of authors, including Becker,[2] Collins,[3] van Geuns,[4] Parsons,[5] and von Sallmann,[6] there has been, to our knowledge, no report up to this time dealing with the healing of an experimental wound of the posterior lens and the sequence of changes which occur when there is free lens material in the vitreous cavity.

Materials and Methods

These experiments were carried out on 35 adult rabbits, under intravenous nembutal anesthesia. After the eye had been rotated with a fixation forceps, a 23-gauge needle, sterilized by heat, was introduced through the sclera posterior to the equator. Under direct observation, a scratch was then made horizontally across the surface of the posterior lens with just enough pressure to rupture the capsule. Streptomycin-penicillin ointment was instilled into the cul-de-sac. The animals were given lethal doses of nembutal at intervals ranging from a few minutes after operation to six weeks later.

After the eyes had been enucleated and the lenses examined grossly, they were fixed in formalin. Sagittal sections were prepared and were stained with hematoxylin and eosin. The periodic acid-fuchsin staining technique was also used as indicated. Ex-

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Fig. 1 (Lyda and Waugh). Section of eye enucleated immediately after traumatic rupture of posterior lens capsule. Note curling, retraction, and thickening of the capsule.

amination of the 70 eyes from the experimental animals was supplemented by the examination of eight unoperated eyes, which were used to establish the normal anatomy and histology of the rabbit eye.

In order to correlate the experimental findings with the findings in human eyes, 89 unselected, consecutive eyes previously sectioned in our laboratory were re-examined from this standpoint.

Experimental results

Gross examination of the experimental eyes showed that some lenses were clear and some opaque. The results of the histologic examinations are most conveniently reported chronologically, according to the period after operation at which the animals were killed.

The eyes of rabbits killed immediately after operation presented under the microscope a rupture of the posterior lens capsule, the edges of which were curled away from the wound and were retracted and thickened (fig. 1). The cortical material was edematous and globule formation was observed in the posterior lens substance.

Examination one hour after operation showed a few mononuclear cells originating in the ciliary body and pars plana and extending along the anterior peripheral vitreous face and zonular fibers toward the lens material (fig. 2).

Examination at six hours revealed an in-

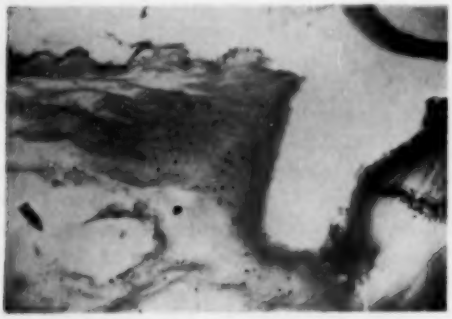

Fig. 2 (Lyda and Waugh). Section of eye enucleated one hour after traumatic rupture of posterior lens capsule. Mononuclear cells that have orignated in the ciliary body are extending along the anterior vitreous face toward the exposed lens material.

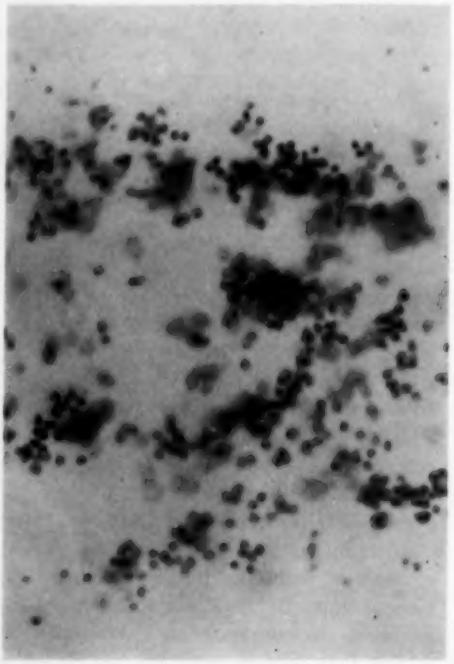

Fig. 3 (Lyda and Waugh). Section of eye enucleated 12 hours after traumatic rupture of posterior lens capsule. Clumps of polymorphonuclear leukocytes are seen in the posterior vitreous near the lens substance.

crease in the number of mononuclear cells originating in the ciliary body and pars plana and extending toward the lens. There was also proliferation of the nonpigmented ciliary epithelium. The lens substance was further disorganized and degenerated and had extruded into the vitreous cavity.

Examination at 12 hours showed the posterior lens capsule thickened, curled, and retracted far from the wound. The lens material was now liquefied and filled the vitreous cavity. Near the ciliary body was an accumulation of mononuclear cells. More centrally, near the denser lens material, were aggregates of polymorphonuclear leukocytes (fig. 3). The retina was edematous and the vessels were engorged. There was also an accumulation of polymorphonuclear leukocytes on the inner retinal surface and in the retinal blood vessels.

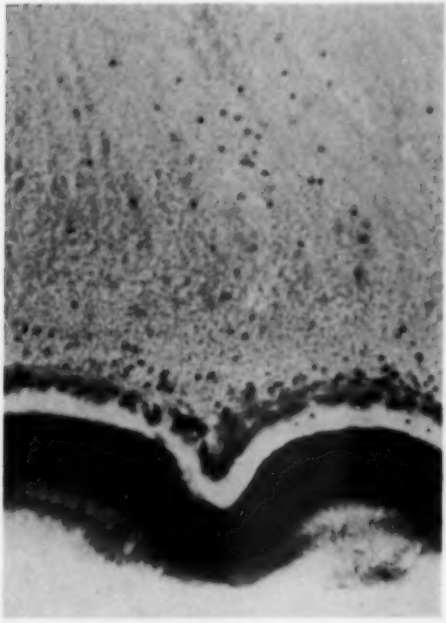

Fig. 4 (Lyda and Waugh). Section of eye enucleated 18 hours after traumatic rupture of posterior lens capsule. Mononuclear and polymorphonuclear cells are exuding from the retina into the vitreous, which is filled with lens fragments.

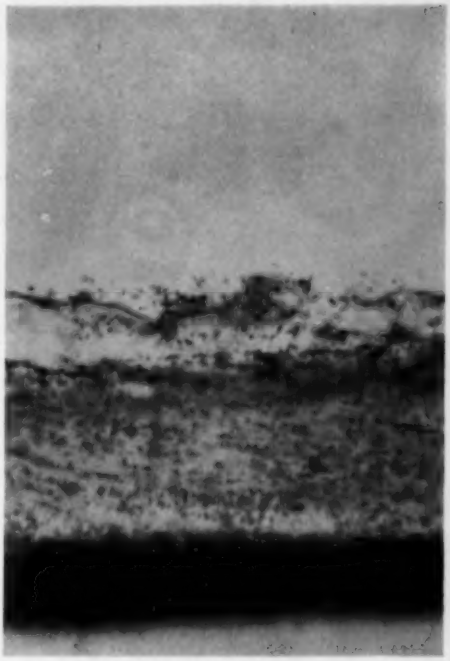

Fig. 5 (Lyda and Waugh). Section of eye enucleated 24 hours after traumatic rupture of posterior lens capsule. Mononuclear and polymorphonuclear cells are exuding from dilated retinal vessels. The inner layers of the retina are edematous.

Examination at 18 hours showed some swelling of the epithelial cells of the anterior lens. A predominantly polymorphonuclear leukocytic response was observed wherever lens material was in contact with the retina (fig. 4). At this time mononuclear cells were more abundant than polymorphonuclear leukocytes in the region of the ciliary body, extending toward the ruptured lens capsule, and throughout the vitreous cavity.

Examination at 24 hours showed a considerable invasion of polymorphonuclear leukocytes into the lens substance, with apparent dissolution of the lens material by these cells. Invasion by mononuclear cells was particularly marked in the region of the ciliary body and the pars plana. The retina, however, showed a layer of edem-

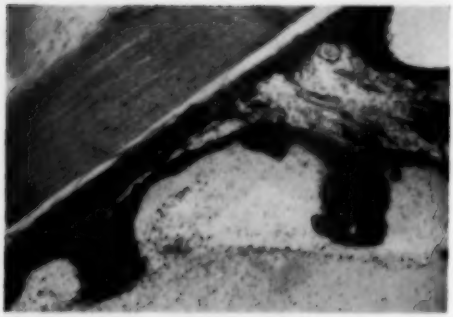

Fig. 6 (Lyda and Waugh). Section of eye enucleated 36 hours after traumatic rupture of posterior lens capsule. Polymorphonuclear leukocytes and macrophages are seen about the ciliary processes and in the chamber angle. Macrophages are observed within the iris and the ciliary body.

atous ganglion cells and an outpouring of mononuclear cells as well as of polymorphonuclear leukocytes (fig. 5).

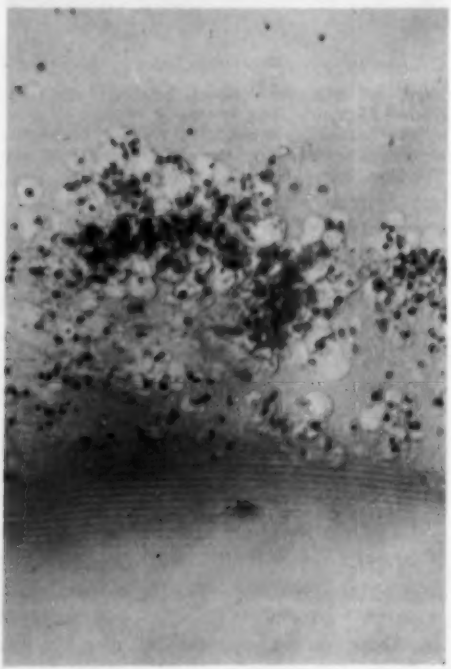

Fig. 7 (Lyda and Waugh). Section of eye enucleated three days after traumatic rupture of posterior lens capsule. Mononuclear cells now predominate and are observed phagocytosing lens substance in the vitreous.

Examination at 36 and at 48 hours also showed a preponderance of polymorphonuclear leukocytes in the extruded lens material along the retinal surface, with a mononuclear cell response chiefly from the ciliary body (fig. 6). Retinal detachment was noted in one of the four eyes examined at this time.

Examination at three days showed a decrease in polymorphonuclear leukocytes and a relative increase in the mononuclear cells (fig. 7). A cellular membrane was present over the face of the vitreous and extended from the pars plana centrally on both sides. This membrane, which apparently originated in the pigment epithelium, was made up of spindle type cells with dark-staining nuclei.

Examination at four days showed the extracellular response to be greatly diminished, only a few mononuclear cells now remaining. At the equator, germinal epithelial cells could be seen proliferating and growing toward the ruptured posterior capsule. The bow equatorial cells were also proliferating posteriorly (fig. 8). The extruded lens substance had diminished in amount and seemed to have been partially absorbed.

Examination at five and at six days showed that all cellular response in the vitreous (fig. 9) and the retina (fig. 10)

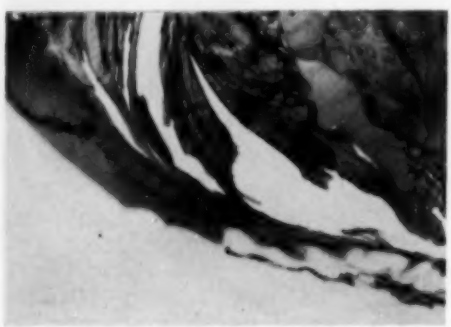

Fig. 8 (Lyda and Waugh). Section of eye enucleated four days after traumatic rupture of posterior lens capsule. There is now posterior proliferation of bow equatorial cells. Proliferating spindle cells continue on the outer side of the ruptured capsule.

Fig. 9 (Lyda and Waugh). Section of eye enucleated five days after traumatic rupture of posterior lens capsule. The mononuclear response in the vitreous is now minimal wherever free lens material is exposed and the posterior proliferation of epithelium has not yet covered the gap in the capsule.

Fig. 11 (Lyda and Waugh). Section of eye enucleated six days after traumatic rupture of posterior lens capsule. Note mononuclear cell exudation and proliferation originating in pars plana. The retina is detached.

had diminished except for a few scattered cells.

Examination of specimens removed over the remaining five weeks of observation showed a minimal cellular response. There was continued development of a membrane originating in the ciliary body (fig. 11). The germinal epithelial cells showed further posterior proliferation along the posterior capsule up to, and around, the curled ends. These cells were a single layer in thickness.

Examination at seven days showed that all the lens material in one of the four eyes examined was extruded. Proliferating an-

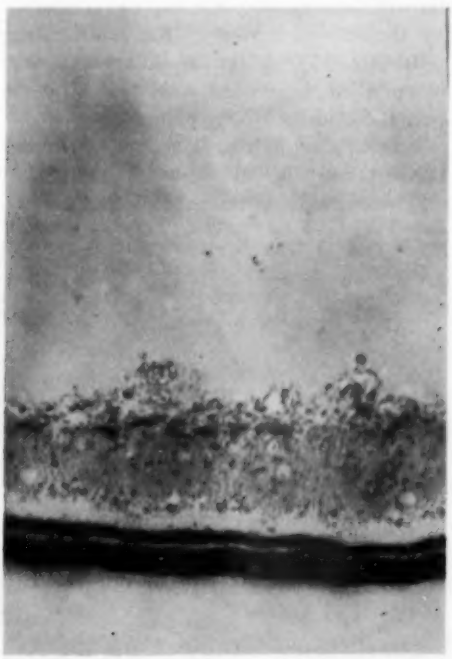

Fig. 10 (Lyda and Waugh). Section of eye enucleated six days after traumatic rupture of posterior lens capsule. There is minimal inflammation wherever the lens-filled vitreous is in contact with the retina. Retinal edema persists.

Fig. 12 (Lyda and Waugh). Section of eye enucleated eight days after traumatic rupture of posterior lens capsule. The small peripheral incision has healed. Note the curled and retracted ends of the posterior capsule and the posterior epithelial proliferations.

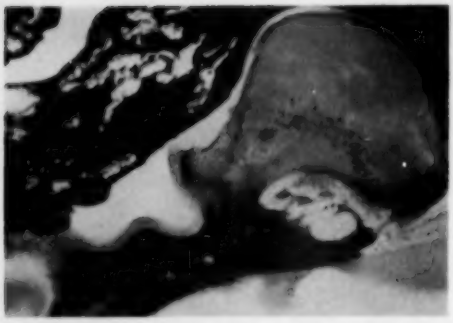

Fig. 13 (Lyda and Waugh). Section of eye enucleated 11 days after traumatic rupture of posterior lens capsule. A Soemmerring ring has formed from germinal epithelial cells.

terior capsular epithelium cells could be seen lining the adjacent posterior capsule. In another eye a large clump of proliferating epithelial cells was observed lying in the degenerated lens substance.

At examination on the eighth day the posterior proliferating germinal epithelial cells were observed to elongate as their growth was followed posteriorly. They became spindle-shaped and took on the appearance of fibroblasts. In one of the eyes examined healing of the traumatic incision was evident. The cells had bridged a small gap in the lens (fig. 12) and were piled up many layers thick in the area of rupture.

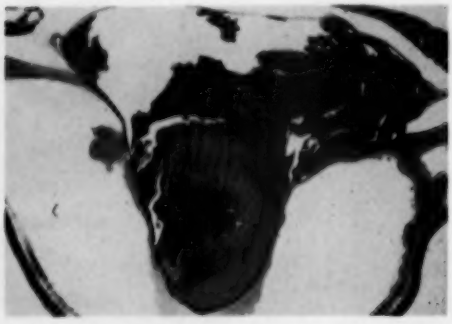

Fig. 14 (Lyda and Waugh). Section of eye enucleated three weeks after traumatic rupture of posterior lens capsule. Proliferating epithelial cells completely surround the herniated posterior lens. Although growth continues under the capsule, this section does not show continuity with germinal or bow cells.

Examination at nine and at 10 days showed essentially the observations just described for examination at eight days.

Examination at 11 days showed the formation of a Soemmerring cataract. Proliferation of germinal cells was plainly evident (fig. 13). A well-developed cyclitic membrane was growing across the vitreous face. Examination at 12 and at 14 days revealed the same findings.

At two and one-half weeks, even when the ruptures were large, the epithelial cells had grown far posteriorly into the vitreous cavity, in an apparent attempt to surround such lens material as was extruded but not completely disorganized.

Examination at three weeks showed bridging of the gap in the capsule by the spindle type of epithelial cells, with continued growth underneath the capsule on the other side of the rupture (fig. 14). One section showed questionable new capsule formation by the more anterior of the proliferating cells (fig. 15). A similar observation was made when the section was stained by the periodic acid-fuchsin technique.

Examination at four, five, and six weeks showed only the following changes:

There was apparent maturation of the

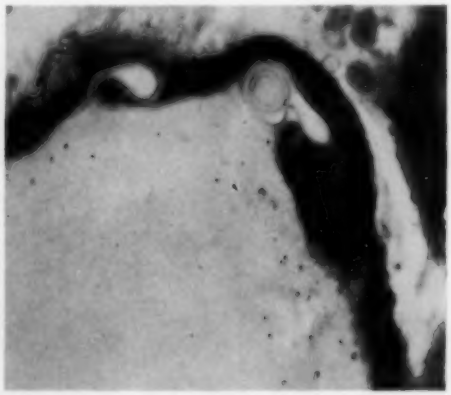

Fig. 15 (Lyda and Waugh). Section of eye enucleated three weeks after traumatic rupture of posterior lens capsule. Capsule like material has been secreted by spindle cells at the edge of the retracted posterior capsule. Spindle cells also cover the herniated lens mass.

spindle cells (fig. 16). The more anterior proliferating cells had become more cuboidal. The nuclei were less darkly stained. There were apparent abortive attempts at capsule formation.

Histologic examination of the experimentally damaged eyes thus revealed three particularly prominent changes:

1. The first alteration in the posterior segment was a response of a few mononuclear cells from the pars plana. As lens material extruded farther into the vitreous and came into contact with the retina, there was a dilatation of the retinal vessels, with an outpouring of polymorphonuclear leukocytes. The ciliary body became engorged, and there was an exudation of mononuclear cells and of some polymorphonuclear leukocytes. The latter invaded the vitreous and infiltrated the lens substance, producing an apparent lysis of lens material. This early response, predominantly polymorphonuclear leukocytic in character, was followed by the appearance of an increased number of mononuclear cells. These cells remained dominant until the fourth day, but by the end of the fifth day all but a few had disappeared. During the remaining five weeks of observation the cell response was minimal (fig. 17).

Fig. 17 (Lyda and Waugh). Section of eye enucleated five weeks after traumatic rupture of posterior lens capsule. Proliferation of inflammatory cells is observed along Cloquet's canal.

2. In the very early stages of observation, examination of the anterior chamber showed a protein coagulum with some macrophages and a few polymorphonuclear leukocytes (fig. 18). The iris and ciliary body showed some edema, dilatation of ves-

Fig. 16 (Lyda and Waugh). Section of eye enucleated five weeks after traumatic rupture of cells along the posterior capsule. Note clumps of epithelial cells in the liquefied cortex.

Fig. 18 (Lyda and Waugh). Section of eye enucleated 12 hours after traumatic rupture of posterior lens capsule. Note cellular exudate in anterior chamber.

sels, and infiltration by plasma cells and macrophages. There was no residual atrophy of the iris or ciliary body after clearing of the infiltration. Changes noted in the choroid were limited to dilatation of the vessels in some sections.

3. Proliferation by anterior lens epithelial cells began at about the fourth day in the germinal epithelium and bow cells, after the cellular response had diminished. Growth was observed posteriorly along the posterior lens capsule to the curled edges of the rupture. As the epithelial cells grew posteriorly, they assumed a spindle shape and tended to become several layers thick. After they had reached the ends of the rupture in the capsule, they piled up and bridged the gap in the form of a membrane several layers thick. The cells also showed a distinct tendency to grow around the entire mass of lens material extruded into the vitreous wherever it retained some structure. In all the eyes which showed posterior proliferation, the equatorial cells nearest the break exhibited all the growth. The equatorial cells on the other side of the rupture remained relatively inactive. The proliferating epithelial cells extended across the gap and anteriorly under the posterior capsule of the inactive side.

Discussion

Posterior proliferation of the lens epithelium from the bow epithelial cells rather than from the germinal epithelium was an unexpected finding in the eyes of rabbits in which the posterior lens had been damaged. The bow cells seemed to respond to trauma by growth posteriorly whenever the lens was not sufficiently injured to destroy them entirely. When destruction was extensive, or when loss of lens cortex was sufficient to cause extrusion of bow cells, it was the germinal cells at the equator that extended along the posterior lens capsule.

Posterior growth of germinal epithelial cells in human eyes has been thoroughly reviewed by a number of observers, including Clapp,[7] Friedenwald,[8] Bellows,[9] Samuels,[10] and Cogan, Donaldson, and Reese.[11] Posterior proliferation of the germinal epithelium was noted in the late stages of senile cataract, in cataracta complicata, and in radiation cataract. The histologic findings which these observers described in human eyes seemed to us to be very similar to the posterior proliferation of germinal epithelium which we had observed in rabbit eyes. Although posterior proliferation of bow epithelial cells has apparently not been reported in human eyes, it seems probable that it could occur in human lenses in which cataractous changes were not too extensive.

When the 89 eyes which had been removed on various indications and sectioned in our laboratory were examined from this standpoint, 18 (20 percent) were found to show partial or complete posterior proliferation of bow cells. The amount of posterior proliferation of germinal cells in these eyes was directly proportional to the degree of change in the lens and the extent of associated intraocular disease.

Lenses which were the site of partial cataracts exhibited partial posterior epithelial proliferation. Those which were the site of mature cataracts exhibited, in some cases, proliferation of cells around the whole posterior capsule. The degree of posterior proliferation was also related to the degree of other intraocular changes. Generally speaking, the more extensive the intraocular inflammation, the more extensive was the posterior proliferation of epithelial cells.

In the experimental eyes, the posterior proliferation of bow and germinal epithelial cells was inversely proportional to the early inflammatory cell response. In the first few days after injury, when there was a preponderance of polymorphonuclear leukocytes, no posterior proliferation was noted. Posterior proliferation, in fact, did not occur until all inflammatory cells had greatly diminished in number. The important role which poly-

morphonuclear leukocytes probably played in the changes described was also demonstrated in a rabbit eye which was the site of secondary infection, and in which no posterior proliferation was noted even after 10 days.

These observations suggest that posterior proliferation of lens epithelial cells is a nonspecific response to injury, which may be traumatic, as in these experimental animals, or may be secondary to senile cataract, intraocular disease, or irradiation, as in the human eyes studied.

The time element also seemed to play a part in the type of inflammatory cell response observed in rabbit eyes. A slight initial mononuclear cell response was followed by a marked polymorphonuclear cell response, chiefly from the retinal arterioles in contact with lens substance. During this phase of the reaction polymorphonuclear leukocytes invaded and lysed the lens material. There was then a moderate mononuclear cell response of gradually increasing intensity.

These observations seem to indicate that the type of cellular response which occurs in the posterior segment of the eye is related to the length of time the disorganized lens material remains in the vitreous cavity and to the complexity of the lens protein rather than to any hypersensitivity phenomenon.

Summary

After mechanical disruption of the posterior lens capsule of the rabbit eye, progressive intraocular changes occur, chiefly confined to the posterior segment.

Both the ciliary body and the retina participate in the subsequent lens-induced endophthalmitis. The cellular response appears to be related to the length of time the disorganized lens material remains in the vitreous cavity.

As the cellular response diminishes, the ruptured lens capsule begins to heal. Posterior proliferation of lens epithelial cells permits the coverage of large herniations of extruded lens material.

A comparison of the histologic findings in these experimental eyes with the histologic findings in human eyes which are the site of senile cataract, cataracta complicata, and radiation cataract suggests that posterior epithelial cell proliferation of the lens is a nonspecific response.

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Discussion

Dr. Ludwig von Sallmann (New York): This presentation was very interesting to me, especially with respect to the comparison between the rabbit eye and the human eye.

In the Knapp Laboratory many experiments were carried out in an attempt to elicit proliferation of the lens epithelium by injecting toxic substances into the vitreous of the rabbit's eye. We were unsuccessful in stimulating the cells of the germinative zone and of the lens bow to abnormal migration.

I wonder whether the authors have evidence that experimentally induced inflammatory changes in the posterior segment of the animal eye produced a reaction of the lens epithelium similar to that observed after traumatizing the posterior lens capsule.

Dr. Wood Lyda (New Orleans): The only answer that I can give to Dr. von Sallmann is that in some preliminary experiments that we have done we injected dianine and glycerine into the posterior segment of the eye, much as we ruptured the posterior capsule in these experiments; and even though we noted that there was clouding of the vitreous and that we would get a flare in the anterior chamber and complete clouding of the lens, the changes were all reversible, and the lens would soon clear. We would find a minimal response in the vitreous, and when we examined these few eyes histologically there were no signs of inflammation other than where we had punctured the sclera to go into the vitreous.

HEMATOPOIESIS IN AND AROUND THE EYE*

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and
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For the purpose of determining whether or not any premonitory signs of retrolental fibroplasia are present at birth, 122 sets of eyes† of stillborn infants, and of newborns dying shortly after birth, were selected for study.[1] Excluded from the series were (1) fetuses with a birth weight of less than 300 gm.; (2) all newborn infants whose birth weight and age at death were unknown. The study revealed that, although changes typical of retrolental fibroplasia may be present at birth, they are absent in the large majority of the cases.

A cellular infiltration was also noted in an appreciable number of eyes, particularly in the choroid. As a result of efforts to determine whether these infiltrating cells represented an inflammatory reaction, and if so whether they were related to the development of retrolental fibroplasia, it became apparent that the process was actually hematopoiesis. The phenomenon was observed in 17 of the 122 sets of eyes, or 14 percent, and has now also been observed in two sets of eyes from older infants.

The first blood formation occurs in the yolk sac, and during fetal life many organs may be concerned with it, for example, the liver, the spleen, the lymph nodes, and the thymus. Blood is also formed in connective tissue virtually everywhere over the body, especially in the neighborhood of blood vessels, for example, in the connective tissue of the kidneys and pancreas. Finally, hematopoiesis is taken over by the bone marrow.

Whenever an unusual demand for blood cells arises as in the severe, persistent anemia of congenital syphilis or erythroblastosis fetalis, hematopoiesis may be encountered in many organs in which it does not ordinarily occur. It may also become widespread without any demonstrable cause and has at one time or another been found in virtually every

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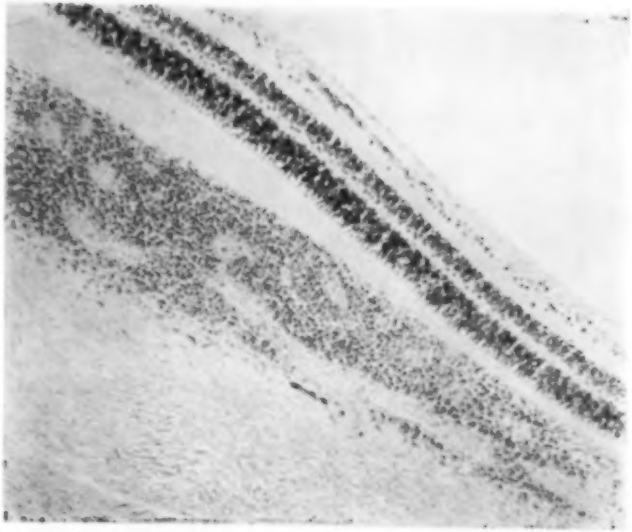
* From the Department of Ophthalmology, Columbia University, College of Physicians and Surgeons, New York. This work has been supported by a grant from the Dunlevy Milbank Foundation, Inc. Dedicated to Prof. Karl Lindner on the occasion of his 70th birthday.

† The sources of these eyes were the Obstetrical and Pediatric Services of the Presbyterian Hospital, New York. The sources of additional eyes are acknowledged elsewhere in the text.

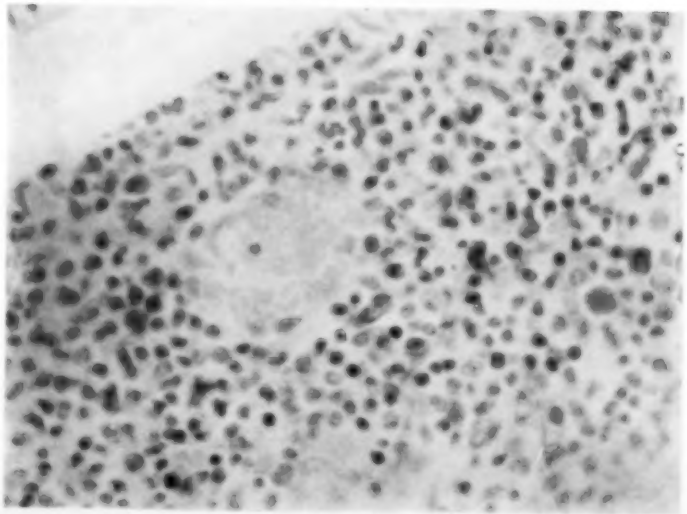


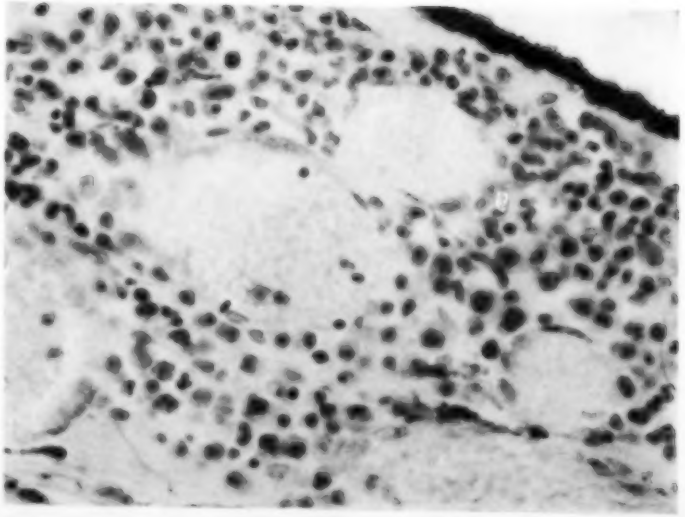
Figs. 3b, 3c, and 4 (Reese and Blodi). Hematopoiesis in and around the eye.

(Fig. 3b). No. 329. A large focus of hematopoiesis in the choroid, with considerable thickening.

(Fig. 3c). No. 329. A large focus of hematopoiesis in the choroid with eosinophilic myelocytes and leukocytes.

(Fig. 4). No. 144. Immature blood cells in the choroid.





organ and tissue of the body, for example, in skin, muscle, appendix, larynx, and breast.[2, 3] From the evidence it is clear that there are many potentially hematopoietic foci and that they are most likely to be found in the region of blood vessels.

In the majority of normal full-term infants, erythropoiesis is limited almost entirely to the bone marrow,[4] extramedullary erythropoiesis diminishing rapidly and soon disappearing after birth even though the need for it may persist. This reduction in extramedullary hematopoiesis may be partly responsible for the progressive anemia of the newborn.

Report of cases

Evidence of hematopoiesis in or around the eye has now been observed in a total of 19 sets of eyes. Twelve of the 19 were from premature infants, seven were from full-term infants. This proportion is not surprising in view of the positive correlation between extramedullary blood formation and fetal immaturity. Of the 12 prematurely born infants, 10 were either stillborn or newborn and two had lived 67 and 107 days respectively. One of the newborn infants had a case of erythroblastosis fetalis; in none of the others was there any demonstrable pathologic cause for the hematopoiesis.

Premature infants

A. *An infant with erythroblastosis fetalis.* This disease shows, characteristically, a widespread extramedullary hematopoiesis due to the severe intrauterine hemolysis.

Case 1. (No. 132*) Male, birth weight, 1,350 gm., lived for 30 minutes. The clinical and pathologic diagnosis was erythroblastosis fetalis. Extramedullary hematopoiesis was found in the liver, spleen, kidney, and thyroid.

Sections of both eyes show numerous small foci of blood formation in both choroids (fig. 1).

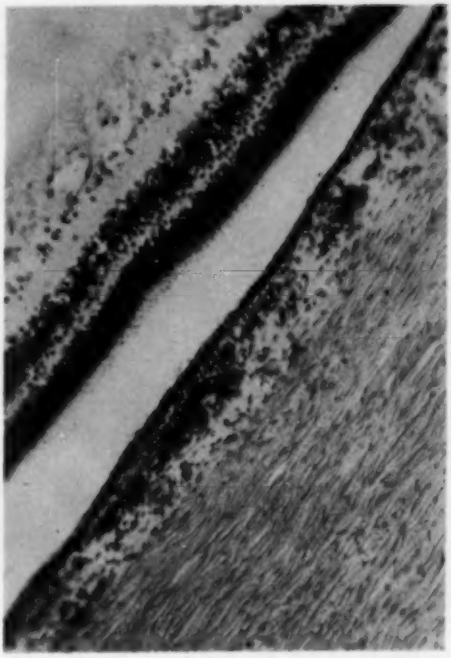


Fig. 1 (Reese and Blodi). No. 132. Small foci of hematopoiesis in the choroid of an infant with erythroblastosis fetalis.

B. *Other stillborn or newborn infants.* The birth weights of the remaining nine premature stillborn or newborn infants were as follows: Less than 1,000 gm., three cases; between 1,000 and 1,500 gm., one case; between 1,500 and 2,000 gm., two cases; and between 2,000 and 2,500 gm., three cases.

Case 1 (No. 106). Male, birth weight 420 gm., stillborn. Widespread hematopoiesis was found in the liver and spleen.

There are a few foci of blood formation in both choroids and in the orbital tissue around the ciliary vessels.

Case 2 (No. 141). Female, birth weight 850 gm., lived for 62 hours and 10 minutes. Intracranial hemorrhages and aspiration of amniotic fluid were found at autopsy. Hematopoiesis was present in liver, spleen, and pancreas.

* This case, and cases No. 178 and No. 186, to be mentioned later, came from the Cornell University Medical College and were obtained through the courtesy of Dr. J. Ellis.

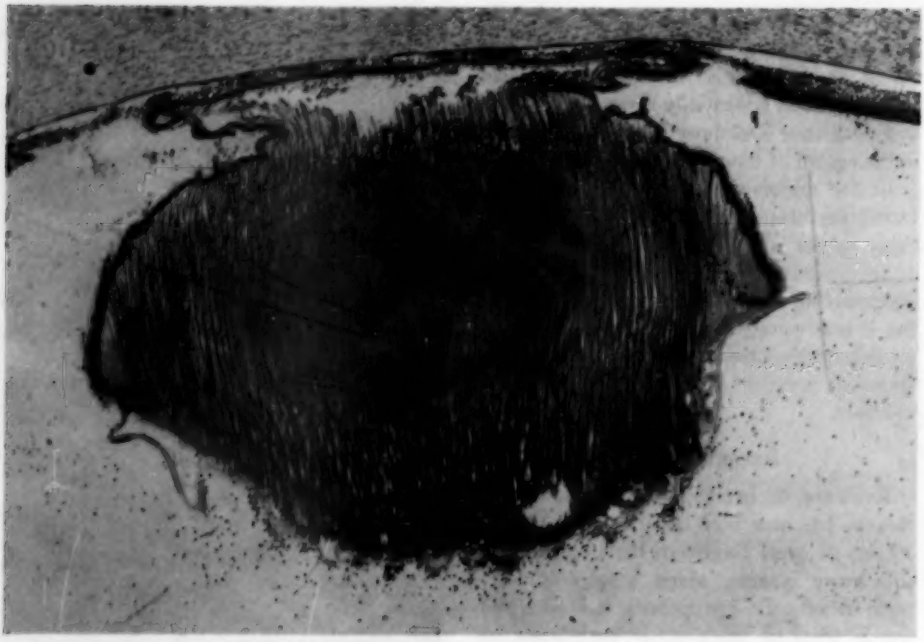


Fig. 2a (Reese and Blodi) No. 141. Immature blood cells infiltrate the iris and are also seen in the pupillary area. In the anterior vitreous, just posterior to the lens, there is a diffuse and marked infiltration of these cells. This illustration was used in a previous article ("Retrolental fibroplasia." Am. J. Ophth., **34**:18, 1951.)

Both eyes show immature blood cells throughout the eye and orbit (figs. 2a, 2b, and 2c).

Case 3 (No. 108). Male, birth weight 790 gm., lived for one hour. There were intracranial and spinal meningeal hemorrhages. Hematopoiesis was found in the spleen and thymus.

A few small areas of immature blood cells are present in the choroid.

Case 4 (No. 171). Female, birth weight 1,420 gm., stillborn. Multiple and extensive hemorrhages were found at autopsy. Hematopoiesis was present in the liver, spleen, and kidney.

Both choroids contain numerous areas of immature blood cells.

Case 5 (No. 84). Male, birth weight 1,630 gm., stillborn. At autopsy hemorrhages were found in the heart, lungs, thymus, and liver. Hematopoietic islands were seen in the spleen.

Both choroids contain a few foci of extramedullary blood formation.

Case 6 (No. 178). Male, birth weight 1,980 gm., lived for six and three-fourths hours. Autopsy showed aspiration pneumonia and pulmonary hemorrhages. Hematopoiesis was found in the spleen.

There are numerous nests of extramedullary hematopoiesis in both choroids.

Case 7 (No. 2). Male, birth weight 2,250 gm., stillborn. Internal hydrocephalus and microgyria were found at autopsy.

There are a few foci of immature blood cells in the left choroid.

Case 8 (No. 277). Female, birth weight 2,310 gm., lived for 61 hours and 35 minutes. On the third day a severe anemia was found with a hemoglobin of 6.5 gm. and 1.7 million

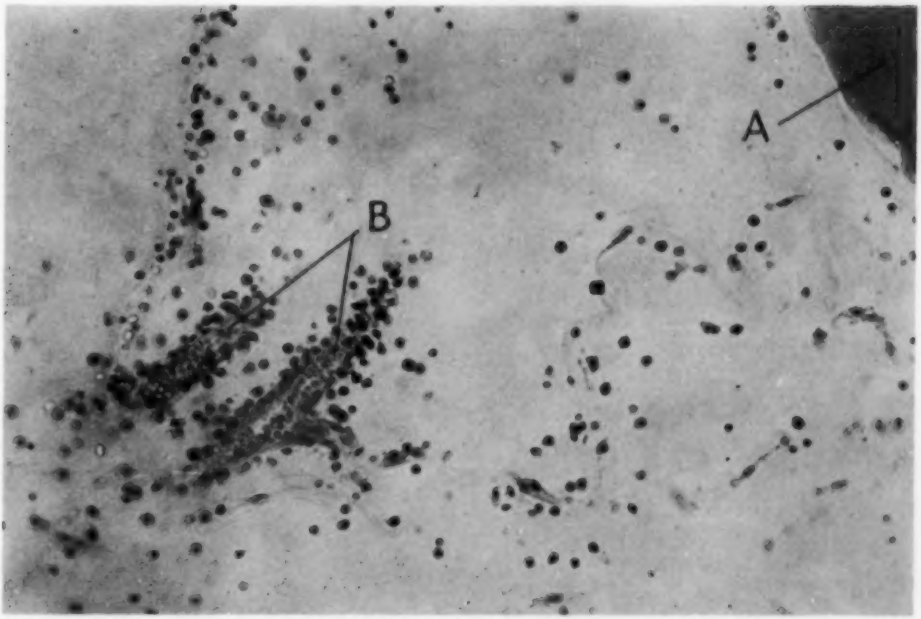


Fig. 2b (Reese and Blodi). No. 141. Immature blood cells in the primary vitreous. (A) Lens. (B) Hyaloid artery.

R.B.C. A transfusion was performed. Hemorrhages and laceration of the tentorium were found at autopsy. There was active hematopoiesis in the liver, spleen, pancreas, and kidney.

Both choroids contain a few areas of blood formation.

Case 9 (No. 34). Female, birth weight 2,400 gm., lived for 18 and one-half hours. A confluent lobar pneumonia was found at autopsy. Blood formation was seen in the liver, spleen, and thymus.

There are large areas of hematopoiesis in both choroids.

C. *Older infants.* Foci of hematopoiesis were found in the eyes of two premature infants who died after 67 and 107 days respectively. The eyes were examined histologically because of bilateral incipient retrolental fibroplasia.

Case 1 (No. 329). Male, birth weight 1,021 gm., died after 67 days. This case was previously reported as an instance of early retrolental fibroplasia. The baby did very poorly after birth. He had frequent attacks of apnea and a persistent and rather severe anemia. He was given five transfusions. The hemoglobin came down to 6.5 gm. on his 53rd day, but after three transfusions on the day of his death, he had 3.36 million R.B.C. and 10.0 gm. hemoglobin. His weight at death was 1,580 gm.

The autopsy revealed the following: (1) An abscess of the scalp; (2) casts in the renal tubules; (3) calcium deposits in the myocardium, orbit, and kidneys, and around the esophagus, thyroid, and thymus; (4) early retrolental fibroplasia; (5) necrosis of adrenals (ACTH had been administered); and (6) lobular pneumonia. Foci of hematopoiesis were found in the cortex and the medulla of the kidneys.

Around the ciliary vessels of both orbits are many nests of immature blood cells (fig.

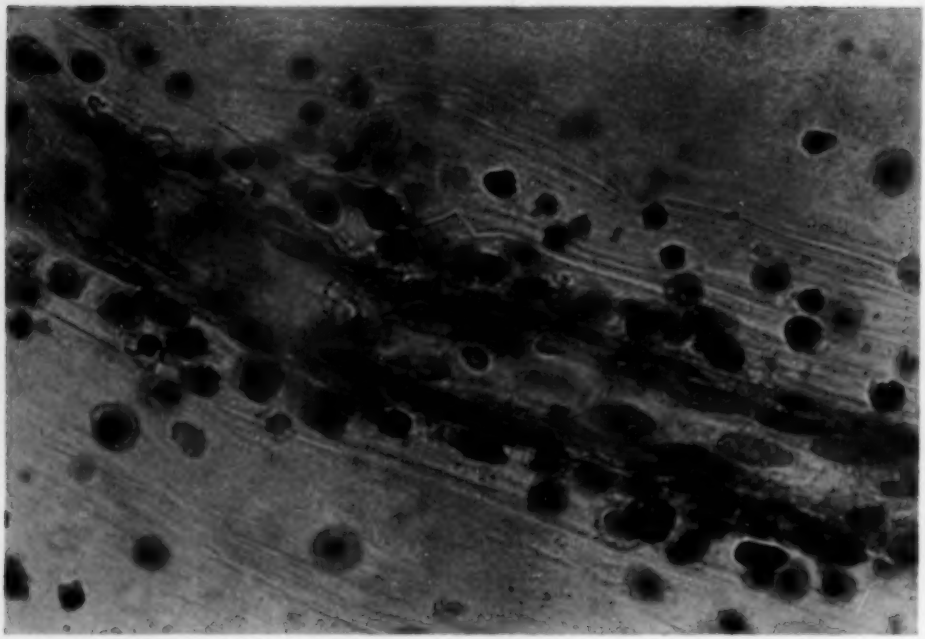


Fig. 2c (Reese and Blodi). No. 141. Immature blood cells around the hyaloid artery in the primary vitreous.

3a), and in the left choroid near the posterior pole there is a large focus of hematopoiesis (fig. 3b). This dense accumulation of immature cells has produced a tumorlike thickening of the choroid like the thickenings that have been described in connection with other organs. Among the premature blood cells, eosinophilic myelocytes and segmented forms with eosinophilic granulation are especially conspicuous (fig. 3c).

CASE 2 (No. 5945).* Female, birth weight 1,134 gm., died after 107 days. There was a persistent anemia and the child received one transfusion. On her 63rd day, when the last blood count was made, she had three million R.B.C. and the hemoglobin was 66 percent of the normal value.

Three weeks after birth the fundi were examined through dilated pupils. Details could not be seen due to a heavy haze in the vitreous. One week later a few vessels could be indistinctly seen. Twelve days later retinal vessels were still indistinct but dilatation and tortuosity could be seen. Three weeks later (12 weeks after birth) the vitreous was clear and a large retinal fold could be seen in the temporal portion of the fundus. This was classified as Grade III, active retrolental fibroplasia. The patient died of suffocation after aspiration of formula.

Microscopic examination of the eyes showed in the choroid several large areas of immature blood cells with myelocytes, pyeloblasts, and nucleated erythrocytes.

FULL-TERM INFANTS

There were seven infants in this series with birth weights of more than 2,500 gm.

CASE 1 (No. 167). Male, birth weight 2,530 gm., stillborn. Autopsy revealed a congenital hydrocephalus with subdural and intraventricular hemorrhages. Hematopoiesis was present in the spleen.

* This set of eyes was obtained by the courtesy of Dr. W. M. Schardt, Manchester, Connecticut, who also furnished all the clinical data.

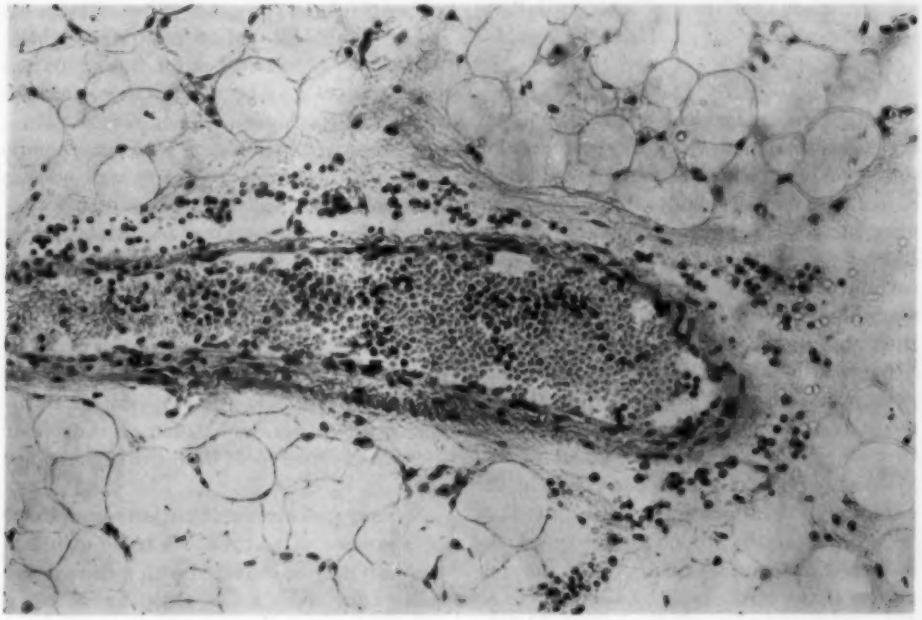


Fig. 3a (Reese and Blodi). No. 329. Immature blood cells around a ciliary vessel in the orbit.

The left choroid contains a few nests of immature blood cells.

CASE 2 (No. 214). Male, birth weight 2,750 gm., stillborn. Evidence of aspiration of amniotic fluid was found at autopsy. Active hematopoiesis was present in liver and spleen.

There are a few foci of blood formation in the choroid of the right eye.

CASE 3 (No. 169). Male, birth weight 2,920 gm., lived for 45 and one-half hours. Autopsy revealed congenital atelectasis of the lungs and hemopericardium. Hematopoiesis was present in the liver.

There are a number of hematopoietic areas in both choroids.

CASE 4 (No. 198).* Female, birth weight 3,067 gm. Multiple hemorrhages of the lungs were found at autopsy. Blood formation was present in the liver.

There are a few hematopoietic foci in the choroids of both eyes.

CASE 5 (No. 144). Female, birth weight 3,150 gm., lived for 60 hours. On the third day the hemoglobin was 15 gm. (104 percent) and the R.B.C. numbered 4.86 million. Necrosis of the myocardium and cardiac dilatation were found at autopsy. Hematopoiesis was seen in the liver and thymus.

Both choroids contain large areas of immature blood cells with nucleated erythrocytes and myeloblasts (fig. 4).

CASE 6 (No. 173). Male, birth weight 3,250 gm., stillborn. The autopsy revealed aspiration of amniotic fluid, congenital atelectasis of the lungs, and hemorrhages in the falx and tentorium. Hematopoiesis was present in the liver, spleen, adrenals, and testes.

There are a few nests of immature blood cells in the choroid of the left eye.

CASE 7 (No. 186). Male, birth weight 4,020 gm., stillborn. The autopsy diagnosis was anoxia in utero. Hematopoietic activity was found in the liver and spleen.

* This set of eyes came from the Margaret Hague Maternity Hospital, Jersey City, New Jersey and was obtained through the courtesy of Dr. N. M. Alter.

Large and small foci of extramedullary blood formation are present in both choroids.

Discussion

Blood-forming faculties in and around the eye, never before described,* were demonstrable in 14 percent of our unselected series of stillborn and newborn infants. The infiltrations were noted primarily in the choroid; the foci were usually small- to medium-sized but occasionally assumed tumorlike proportions (figs. 3b, 3c, and 4). By virtue of its mesodermal origin and its richness in blood vessels, the choroid would seem to be a natural site for ectopic hematopoiesis. Infiltration of immature blood cells, stemming apparently from remains of the hyaloid system, have also been seen in the vitreous. Blood formation was also noted in the orbit, especially around the ciliary vessels, in three cases, and was observed in the connective tissue of the orbit by Gilmour[5] in an 18-mm. embryo but not in embryos of 170 to 190 mm. in length.

The frequency (14 percent) with which hematopoietic foci were found in the eyes of stillborn and newborn infants in this series suggests that the choroid may be one of the physiologic sites of hematopoiesis during fetal life. In only one case could a pathologic cause (erythroblastosis fetalis) be uncovered.

The ocular hematopoiesis in the two older children (aged 67 and 107 days) may have been due to (1) the extreme prematurity of the children, which could account for an immaturity of the hematopoietic process, and (2) the persistent anemia, which could have stimulated blood formation at ectopic sites.

The hematopoietic faculties of the eye may have clinical significance in the following relationships:

1. When the infiltration of the choroid is extensive (figs. 3b, 3c, and 4), it is possible and even probable that sequelae occur which can later be appreciated in the fundus. What these changes may be is, of course, entirely a matter of speculation.

2. The vitreous clouding seen occasionally in premature infants may very well be due to hematopoietic cells (figs. 2a, 2b, and 2c). The degree of haziness may be such as to prevent a view of fundus details for five or six weeks after birth but in the course of some two months or more the vitreous clears.† There are those (Guy[6]) who believe this vitreous clouding in premature infants pressages the development of retrolental fibroplasia. It is our feeling that the vitreous haze could be due to hematopoiesis.

3. The myelocytes and nucleated red blood cells present in the uvea as a result of hematopoiesis may be confused with inflammatory cells. They were so regarded by us when first seen, and Friedenwald,[7] in his description of ocular changes in fetal syphilis, speaks of myeloid cells, myeloblasts, myelocytes, and nucleated red blood cells as the prevailing reaction in the uveas of eight premature infants dying at birth or shortly thereafter; in an additional full-term infant dying one and one-half days after birth, only the usual inflammatory cells were seen. In these cases the maternal Wassermann was positive in five, the infant's Wassermann was positive in two, and the father's and mother's Wassermann reactions were negative in two; in another case the father had had an incomplete course of antiluetic treatment and the Wassermann reaction was unknown.

In his report, Friedenwald was inclined to believe "that in the immaturity of the blood-forming organs in fetal syphilis only a small number of mature wandering cells are available to respond in an inflammatory reaction and that the deficit is made up by an outpouring of immature myeloid cells." From

* Except for instances of hematopoiesis in heterotopic bone marrow. Böck has shown that blood-forming faculties are manifested by the bone in old degenerated globes in patients suffering from severe anemia.

† C. *Older infants.* Case 2 (No. 5945).

the description of the cells and the fact that eight of the nine infants were premature, it would seem to us that the uveal infiltration, in most of the cases at least, represented hematopoiesis. In a personal communication Friedenwald has expressed the same opinion.

4. Uveal infiltration and the resulting fundus changes seen ophthalmoscopically in myelogenous leukemia may be due to activation or resumption of the hematopoietic properties of the uvea rather than to a simple infiltration. It is thought that ectopic hematopoietic sites elsewhere become reactivated in the course of a myelogenous leukemia and it seems logical to assume that a uveal site might be similarly reactivated.

73 East 71st Street (21).
University Hospitals.

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Discussion

Dr. Kenneth C. Swan (Portland, Oregon): Has Dr. Reese observed histologic changes in the pigment epithelium overlying the areas of hematopoiesis? I ask the question because in our routine studies of children in the premature nursery, we have noted ill-defined areas of increased density of the pigment epithelium in association with fine vitreous opacities.

I wonder if the condition which he has described might fit this or any other clinical picture.

Dr. Frank Vesey (Schenectady): I would like to ask Dr. Reese whether he found this hematopiesis existing in infants that were not premature.

Dr. Howard H. Patt (Baltimore): I would like to ask if the infants that showed the extramedullary hematopoiesis showed evidence of this in other organs, more so than do premature infants routinely.

Dr. Frederick C. Blodi (Iowa City, Iowa): Dr. Swan, I will have to say no. Dr. Vesey, I presume you meant the extramedullary hematopoiesis. Yes, it has been noticed in full-term children; as Dr. Reese pointed out, nine of these cases were in full-term children. It can be observed in children up to more than three months of age.

Dr. Patt, many of the other organs at autopsy showed extramedullary hematopoiesis, but there was no significant increase over other children who had ocular foci of hematopoiesis. I don't believe there is a significant relation.

That it could be found is due only to the fact that we looked very carefully through the histologic studies. All this study shows is that morphologic pathology still may reveal some things that we didn't anticipate.

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TWENTY-SECOND MEETING

of the

Association for Research in Ophthalmology, Inc.

Proceedings

Business Session

Auditors' Report

New Members

Directory of Members

Geographical List

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New York, New York

June 1-4, 1953

BUSINESS MEETING

The opening session of the annual meeting of the Association for Research in Ophthalmology, held at the Einhorn Auditorium, Lenox Hill Hospital, New York, convened at 1:10 P.M., Dr. Brittain Payne, chairman, presiding.

CHAIRMAN PAYNE: Members of the Research Association, Ladies and Gentlemen, Distinguished Guests: It is my privilege as chairman of the Research Association Board of Trustees and as a director of ophthalmology of Lenox Hill Hospital to welcome you to New York City, and especially to the Einhorn Auditorium of the hospital for the initial meeting of our national organization.

This auditorium has served in the past on several occasions as the district meeting place for the Eastern Seaboard. The board of governors, the superintendent, and the medical board of Lenox Hill Hospital have always welcomed this group and provided every facility that they could to make the meeting successful. And so, speaking for them, I wish to welcome all of you.

I would like to ask Dr. Tom Travers to stand. Dr. Travers is here from Australia. We want to welcome you to our meeting, Dr. Travers (applause).

CHAIRMAN PAYNE: The first order of business is the report of the secretary-treasurer, Dr. Allen.

SECRETARY ALLEN: Mr. Chairman, the financial report is contained in a report from the auditors, copies of which are available for examination by the members. (See Auditors' Report, page 225).

In addition, the secretary wishes to report that, by action of the board of trustees, the Association for Research in Ophthalmology has invited all eye organizations, both medical and lay, to send representatives to a joint meeting, for the purpose of organizing a central committee or council for research in eye diseases and the prevention of blindness. The primary purpose of this group will be to promote public relations and public information in regard to the great disparity between the expenditure of approximately 150 million dollars for the maintenance of the blind in this country, as contrasted to one million dollars or less for research in ophthalmology.

CHAIRMAN PAYNE: The chairman takes this moment to congratulate Dr. Allen on building up the membership, from approximately 325, which we had at the time I turned the books over to him in 1947, to its present membership of 670. I believe it is a great tribute to his energy and the hard work he has done. I thought I had gained quite a bit over the time when I was assistant secretary and then secretary, but he has more than doubled anything I did.

At this point I will ask for the report of the auditing committee, by Dr. Leopold, before we accept the report of the secretary-treasurer.

DR. LEOPOLD: I have read this report and, as well as I can understand it, I find it completely satisfactory. I recommend that it be approved by the group.

CHAIRMAN PAYNE: You have heard the motion. It is seconded by Dr. McDonald of Philadelphia. Are there any remarks?

(The motion was put to a vote and was carried unanimously.)

CHAIRMAN PAYNE: The next order of business is the report of the nominating committee. Dr. McGavic.

DR. MCGAVIC: The nominating committee respectfully submits the name of Dr. Hunter Romaine of New York for trustee, and the name of James H. Allen of New Orleans for secretary.

CHAIRMAN PAYNE: Are there any nominations from the floor?

DR. KINSEY: I move that nominations be closed and that they be elected.

DR. LEINFELDER: Second the motion.

(The motion was put to a vote and was carried unanimously.)

CHAIRMAN PAYNE: They are elected.

The next order of business is the report of the trustees, by Dr. McDonald.

DR. MCDONALD: Mr. Chairman, this is not a report of the activities of the board of trustees, but at a meeting the other day it was the unanimous opinion of the trustees that Dr. Arnold Knapp, because of his many years of service to ophthalmology, be recommended for election as an honorary member of this society.

I move you, Mr. Chairman, that Dr. Arnold Knapp be elected an honorary member of the Association for Research in Ophthalmology.

DR. MCGAVIC: I second that motion, sir.

(The motion was put to a vote and was carried unanimously.)

CHAIRMAN PAYNE: If there is no further business at this time, I will entertain a motion for adjournment of the executive session.

DR. LEINFELDER: I so move.

DR. SWAN: I second.

(The motion was put to a vote and was carried unanimously.)

DR. SWAN: Mr. Chairman, I wonder if you would entertain a motion calling for a standing vote of thanks for your long period of service to the association

SECRETARY ALLEN: I will usurp the Chair for a moment. I believe everyone heard the motion. Dr. Swan has moved that we extend a rising vote of thanks to Dr. Payne for his long, hard arduous work for the society. This is a very deserving motion. I would like to second it myself. Do we hear a second from the floor?

(The motion was severally seconded, was put to a vote and was carried unanimously, and the audience arose and applauded.)

CHAIRMAN PAYNE: It has been a pleasure. Thank you very much.

AUDITORS' REPORT

Association for Research in Ophthalmology, Inc.

To the Members of the Board of Trustees
Association for Research in Ophthalmology, Inc.
New Orleans, Louisiana

Gentlemen:

Scope of examination

We have examined the cash basis accounts of the secretary-treasurer of the Association for Research in Ophthalmology, Inc., as of December 31, 1952, and for the year then ended. Our examination was made in accordance with generally accepted auditing standards, and accordingly included such tests of the accounting records and other such auditing procedures as we considered necessary in the circumstances, except that we did not confirm unpaid dues directly with members.

Certificate

In our opinion, subject to limitation of the scope of our examination as set forth in the preceding paragraph, the accompanying statements present fairly the fund balances of the Association for Research in Ophthalmology, Inc., as of December 31, 1952, and the total receipts and disbursements for the year then ended, in conformity with generally accepted accounting principles applied on a basis consistent with that of the preceding year. We do not feel that the limitation referred to above is sufficiently important to negate this opinion.

Very truly yours,
B. B. Woolley & Co.,
New Orleans, Louisiana,
May 21, 1953.

Cash and Securities in Funds
December 31, 1952

	General Fund	Proctor Medal Fund	Total
Cash:			
Cash in bank	$1,480.52	$ 15.37	$ 1,495.89
Cash for deposit	493.00	—	493.00
	$1,973.52	$ 15.37	$ 1,988.89
Securities:			
U. S. Treasury Bonds[1]	—	10,184.63	10,184.63
	$1,973.52	$10,200.00	$12,173.52

[1] At Cost—2½%—due 1967-72. At December 31, 1952, coupons amounting to $250.00, matured in 1952, were affixed to the bonds.

Statement of Cash Receipts and Disbursements
Year Ended December 31, 1952

		General Fund	Proctor Medal Fund
Cash Balance January 1, 1952		$1,735.05	$15.37
Add—Receipts:			
Prior years' dues:			
9 Active members @ $5.00	$45.00		
1 Educational member @ $2.00	2.00		
1 Sustaining member @ $25.00	25.00	$ 72.00	
1952 Dues—Page 4		3,034.00	
1953 Dues			
1 Active member @ $5.00		5.00	
Banquet proceeds		410.00	
Bond interest		250.00	
Total Receipts		$3,771.00	—
		$5,506.05	$15.37
Deduct—Disbursements:			
Convention expenses:			
Dinners	$628.83		
Programs, mailing fees, notices	211.93		
Expense of Secretary-Treasurer	250.00	$1,090.76	
Publication—American Journal of Ophthalmology		1,000.00	
Stationery, supplies and printing		253.91	
Auditing		133.00	
Postage		85.84	
Insurance—$5,000.00 position bond—Secretary-Treasurer		25.00	
Safety deposit box rental		9.60	
Cash award for best 1951 convention paper		100.00	
Telephone		9.34	
Salaries—gross	$647.00		
Less—Withholding and FICA tax withheld	138.25	508.75	
FICA and withholding taxes paid		[1]172.50	
Dues paid International Congress of Ophthalmology		100.10	
Bank charge		4.25	
Contribution		25.00	
Sundry		14.48	
Total Disbursements[2]		3,532.53	
Cash Balance December 31, 1952		$1,973.52	$15.37
Composed of:			
Cash in bank		$1,480.52	$15.37
Cash for deposit		493.00	—
		$1,973.52	$15.37

Notes:

[1] FICA and withholding taxes for October and November, 1952, amounting to $23.00 unpaid at December 31, 1952.

[2] Proctor medal for 1952 costing $146.90 was unpaid at December 31, 1952, and is not provided for in the above statement. This was paid on April 22, 1953.

CHANGES IN MEMBERSHIP AND RECONCILIATION WITH DUES PAID
Year Ended December 31, 1952

Changes in Membership	INACTIVE	LIFE	HONORARY	EDUCATIONAL	ACTIVE	SUSTAINING	TOTAL
Paid-up Membership January 1, 1952	-0-	1	9	78	444	24	556
Unpaid Membership January 1, 1952 (see text)	-0-	-0-	-0-	11	60	3	74
Membership January 1, 1952	-	1	9	89	504	27	630
Add:							
Elected for 1952 Membership at October, 1951, meeting of board of trustees				5	18		23
Elected for 1952 Membership at June, 1952, meeting of board of trustees				5	24		29
Elected to Honorary Membership at June, 1952, meeting of board of trustees (transferred from active)			4		(4)		-0-
Total additions	-	-	4	10	38	-	52
Deduct:							
Deceased in 1952			1		5	1	7
Resigned in 1952					3		3
Total deductions	-	-	1	-	8	1	10
Changes in Classes of Memberships:							
From active to sustaining					(12)	12	
From sustaining to active					5	(5)	
From educational to active				(11)	11		
From educational to inactive	1			(1)			
Net changes	1	-	-	(12)	4	7	-
Membership December 31, 1952	1	1	12	87	538	33	672
Reconciliation with Dues Paid:							
Dues for 1952 Paid in 1951				1	1		2
Dues Paid in 1952 for 1952:							
Inactive, life and honorary waived	1	1	12				14
Educational @ $2 each $ 74.00				37			37
Active @ $5 each 2,160.00					432		432
Sustaining @ $25 each 800.00						32	32
$3,034.00	1	1	12	38	433	32	517
Add:							
Unpaid membership December 31, 1952	-0-	-0-	-0-	49	111	2	162
	1	1	12	87	544	34	679
Less:							
Three honorary members elected in 1952 paid 1952 active dues					3		3
One sustaining member who died in 1952 paid 1952 dues						1	1
Unaccounted for difference					3		3
	-	-	-	-	6	1	7
	1	1	12	87	538	33	672

SUMMARY OF MEMBERSHIP BY YEARS

To December 31, 1952

Years Ended December 31	Total Members	Years Ended December 31	Total Members
1952	[1]672	1940	270
1951	[2]556	1939	268
1950	[2]509	1938	272
1949	[2]474	1937	249
1948	422	1936	240
1947	306	1935	245
1946	324	1934	230
1945	[3]—	1933	219
1944	283	1932	203
1943	[3]—	1931	193
1942	281	1930	134
1941	279		

NOTES: [1] Represents official membership, whether dues were paid or unpaid for current and prior years.
[2] Includes only members whose dues were paid in full to date.
[3] Not available, due to wartime dislocation.

HISTORY

The association was incorporated on July 20, 1936, under the laws of the state of New York. However, it had been an unincorporated group for some years earlier, operating under a constitution and related by-laws, which were embodied in the certificate of incorporation. The Association has no shareholders and is exempt from federal, state, and local taxes. However, it is required to file a federal information return reporting the source and disposition of income annually.

COMMENTS

We found matured coupons amounting to $250.00 attached to the U. S. Treasury bonds, representing interest for the calendar year 1952. These were detached in the presence of our representative and deposited in bank on May 15, 1953. Coupons for prior years had previously been clipped.

At the meeting of the board of trustees in June, 1952, the board directed that the $250.00 annual interest income from the Proctor Medal Fund be deposited henceforth in the General Fund and that the cost of the annual Proctor medal be paid out of the General Fund. We have, therefore, presented the interest income in that manner in the Statement of Cash Receipts and Disbursements on page 226. It should be noted that no disbursement is shown for the medal in 1952, its cost having been paid on April 22, 1953.

There was no material change in either the total receipts or disbursements for the year, compared with 1951.

Regarding changes in membership in the current year and total membership by years, it should be noted that both statements include members for 1952 whose dues were in arrears. Total membership for the three prior years had included only members whose dues were paid to date. As a result, the change in presentation increased the membership at the end of 1952 by 162.

We did not confirm dues in arrears directly with members but satisfied ourselves by other means that the total of 162 was substantially correct at December 31, 1952.

According to information given us orally by the secretary-treasurer, it is the intention of the board of trustees, governing body of the association, to retain members whose dues are delinquent until the board itself orders that they be dropped for nonpayment. The constitutional provision remains unchanged which provides that new members, upon being approved by the admissions committee and the board of trustees, must pay dues for the first year to attain membership.

We examined minutes of the board of trustees and other pertinent data and observed that the constitutional provisions requiring bonding of the secretary-treasurer and an annual audit by a certified public accountant had been met.

The records were found to be well kept and the financial transactions of the year properly authorized. Please accept our sincere thanks for the courtesy and assistance given us during the engagement.

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1953

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Alberstadt, Norbert F., 921 Commerce Bldg., Erie, Pa.
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B

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Barkan, Hans, 2400 Clay St., San Francisco 15, Calif.
Barkan, Otto, 490 Post St., San Francisco 2, Calif.
Barnett, Irving F., 30 N. Michigan Ave., Chicago 2, Ill.
Barrere, Luciano E., Acisclo Villaran 356, Lima, Peru.
Bassen, Edward J., 70 E. 66th St., New York, N.Y.

[1] Attended 1953 Meeting
[2] Honorary Member
[3] Life Member
[4] Sustaining Member
[5] Educational Member

Bedell, Arthur J.,[3] 344 State St., Albany, N.Y.
Beetham, William P., 108 Bay State Rd., Boston, Mass.
Bell, Dudley P., 411 30th St., Oakland, Calif.
Bell, Richard P., Jr., 11621 Detroit Ave., Cleveland 2, Ohio.
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